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BIOLOGICAL BULLETIN

PUBLISHED BY

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THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Mass. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk:

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees except that Trustees *Emeriti* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory

Gentlemen:

I submit herewith the report of the seventy-fifth session of the Marine Biological Laboratory.

1. Housing

The Laboratory still faces a serious problem in providing housing for investigators, students and research assistants despite the building of thirty cottages on the Devil's Lane Tract and the purchase of houses in the immediate vicinity of the central campus. During the past winter a topographical survey of the unused part of the Devil's Lane Property has been completed and the prospective locations of access roads have been mapped. The Laboratory must take immediate steps to provide additional cottages to be available for occupancy in 1964.

2. Systematics-Ecology Program

This program, under the direction of Dr. Melbourne R. Carriker, is now well under way. Plant modifications were made in a selected area of the Crane wing to house the research activities of the program. Quarters and service rooms for the George M. Gray Museum have been developed in the Stone Building. In addition to the Ford Foundation Grant, support for this program is being provided by grants from the National Science Foundation, the National Institutes of Health and the Office of Naval Research.

3. Comparative Physiology Training Program

This program will be instituted in 1963 under the direction of Dr. C. Ladd Prosser. The staff will include Dr. Benjamin Zweifach and Dr. Lewis H. Kleinholz.

4. Personnel Changes

Because of the pressure of other activities, particularly the Indian Ocean Survey, Dr. John H. Ryther found it necessary to resign as head of the training program in Marine Ecology, though he will continue to function as a member of the staff. Dr. W. R. Taylor will succeed Dr. Ryther as head of this program.

5. *Biological Collecting*

This past summer the Captain Bill III, Klimm, Master, was chartered as a collecting boat, particularly for squid and bottom fish. This proved a most successful venture. There was a constant and adequate supply of squid for the large number of investigators who use this form, and a variety of additional problems were made possible, by the specialized bottom fish which were obtained in numbers. Included were angler fish, sea robins, dogfish, sand-dabs, skates, torpedos, cod, sculpin, bluefish and sturgeon.

6. *Sea Water Well*

The Supply Department has experienced serious difficulty during the winter months in maintaining the live research material collected for shipment to various institutions in this country and abroad. In the past the water for these animals was drawn from the Eel Pond with serious losses of animals following heavy rains or snows. To overcome this a salt water well was sunk adjacent to the Supply Department Building. Sea water was reached at 103 feet. This water has the same salinity as Martha's Vineyard Sound water, maintains a constant temperature throughout the year at approximately 52° F. and, in spite of a low pH, most marine forms, both vertebrate and invertebrate, can be maintained over long periods of time in normal physiological condition. The Laboratory has engaged a consultant to work out methods for raising the pH to normal sea water values without too great an increase in the temperature of the water, the lower temperatures being desired for forms collected from the deeper waters.

7. *Deaths*

It is with sorrow we note the death of Lawrason Riggs on January 6, 1963. Lawrason Riggs served the Laboratory with distinction for nearly 40 years, first as Treasurer (1924–1942) and then as President of the Corporation (1942–1952). This included the periods of the depression and World War II when the Laboratory faced difficult financial and operational problems. That the Laboratory weathered this critical period as well as it did was due in no small measure to the interest and great common sense of Lawrason Riggs.

Respectfully submitted,
Philip B. Armstrong,
Director

1. Memorials

GIOACCHINO FAILLA

by Roberts Rugh

A Corporation Member since 1940 and a Trustee of the Marine Biological Laboratory since 1948, Dr. Failla's life was snuffed out instantly in a head-on collision on the icy roads of Illinois near his laboratory on December 15th, 1961. This accident removed from the modern nuclear world one of the world's leading experts on radiation dosimetry and protection and the man most responsible for radiological physics as a profession. It was a tragic loss of a man who, though retired from his administrative duties, retained the mental vigor and perspective so urgently needed in our atomic age.

Dr. Failla was born in Sicily in 1891, came to New York in 1906, won the Pulitzer Scholarship which put him through the Engineering School at Columbia where he then obtained his M.A. degree. After establishing himself as the physicist of Memorial Hospital, he obtained a leave of absence long enough to procure his D.Sc. degree at the University of Paris under Madame Curie. Early in this period, in 1916, he also became a citizen of the United States.

Memorial statements have been published in Dr. Failla's behalf in RADIOLOGY by Dr. Quimby, in the AMERICAN JOURNAL OF ROENTGENOLOGY, RADIUM THERAPY AND NUCLEAR MEDICINE by Dr. Rossie, in the BRITISH JOURNAL OF RADIOLOGY by Dr. Gray, and in RADIATION RESEARCH by Dr. Marinelli, all world-renowned radiological scientists, and all attesting to Dr. Failla's everlasting contributions to radiology, particularly in the area of radiological physics.

Dr. Failla, an honorary member of the world's most active radiological societies, received many awards and medals, and an honorary degree from the University of Rochester. He was on the first and most of the succeeding Committees on Radiation Units; Standardization and Protection Committees for both the National and International Commissions of Radiation Protection; on the wartime National Defense Committee and Radiological Instrument Panel of the Armed Forces Special Weapons Project; the Advisory Committee on Isotope Distribution, the Advisory Committee on Biology and Medicine of the U.S. AEC and the Genetic Committee; and on the National Academy of Science Committee on the Biological Effects of Atomic Radiation. He served as the Scientific Attaché at the Rome Embassy during the first World War, and was later Consultant to the U. S. Public Health Service, the Veterans Administration and on the main Commissions of both the International Commission for Radiation Protection and the International Commission on Radiological Units. He was the chief writer of the NCRP Handbook #59 which contains the basic philosophy of the most modern radiology on protection against radiation effects. His contributions in radiation physics are known around the world.

We, here at the Marine Biological Laboratory, who were fortunate to know him, will forever be in debt to Dr. Failla for his contributions in the field of *radiobiology*. Among his seventy published papers, ten dealt with the clinical applications of radiation and twenty-four with biological effects. Thus, he was truly a biophysicist, doing his research in the field of nuclear energy. One of his earliest papers, written in 1922 with Dr. Suqiura, dealt with the long-term effects of radiation on mice and their non-irradiated progeny. He was most concerned about delivering effective radiation to malignancies without endangering the life of the patient, the doctor, or the technician, so that his early papers dealt with erythema and tissue susceptibility and with the fundamental effects of ionizing radiations on organisms, tissues, and cells. He formulated one of the first theories as to the biological effects of ionizing radiations, and during his last years, in collaboration with his wife, Patricia McClement Failla, analyzed the process of ageing particularly as it might be related to radiation and carcinogenesis.

Dr. Failla's many contributions in the field of radiological physics and biology will stand the test of time. He was never one to seek publicity, only the truth. Few were aware, however, that he carried to his grave some effects of his early daring in the handling of radium. His hands bore innumerable scars of radiation-cancer, but this in no way deterred his efforts. But Dr. Failla made two major contributions for which we here at the MBL will always benefit. The first grew out of his concern that the various disciplines, represented so well here, should not go the way of such specialization that they become completely divorced from each other. He vigorously championed the interdependence of scientific effort and achievement and in 1952 he, more than anyone else, organized the Radiation Research Society which was designed specifically to guarantee inter-disciplinary cohesion. Its instrument is the journal, RADIATION RESEARCH, and its society membership now numbers well over 1000. This society includes scientists from more than biology and physics. It provides a cross-fertilization, as he put it, of genetics and medicine, physiology and morphology, psychology and ecology, all drawn together by the unifying concern with atomic energy. Life, to Dr. Failla, could not be understood by the dissociation of the various approaches to its study.

Dr. Failla's second major contribution of specific benefit to us here is the cesium-137 unit and the organization of the entire fourth floor of the new laboratory where some 60 scientists are this summer using ionizing radiations as a tool in research. The cesium unit has no duplicate anywhere; it was custom-made for the MBL. It is an example of Dr. Failla's artistry as well as his scientific genius. It is absolutely fool-proof, and can be used without the slightest hazard to anyone, and yet it can deliver very accurate doses of gamma radiation to biological research material for years to come, with a minimum of expense.

Dr. Failla's listed accomplishments cannot reveal the depth or breadth of the man. Few have had more friends, and few have lived to have their contributions to society more recognized, yet few have been more humble. He mingled with the greatest, was consulted by the most brilliant scientific minds, and yet he had time and sympathetic consideration for the rest of us. While he was trained as a physicist, I believe he would like to be remembered for his contributions in biology, not the least of which were made here at the Marine Biological Laboratory.

FRANZ SCHRADER

by DONALD E. LANCEFIELD

Franz Schrader was born in Magdeburg, Germany, March 11, 1891, and came to this country with his family at the age of 12.

As a boy he made his first insect collections on vacations in the Harz Mountains. His interest as a naturalist continued and grew while he lived on Staten Island. At this time he also became an expert sailor and ardent fisherman, interests which continued throughout his life.

He entered the School of Mines of Columbia University in 1910, but shifted to Columbia College after two years to follow his major interest in zoology. After his graduation in 1914, he spent two years of research with the U.S. Bureau of Fisheries, with an additional year as Chief Pathologist, following graduate study at Columbia. He received his Ph.D. under Professor E. B. Wilson in 1919. The following year he married Sally Hughes, who was also a student of Professor Wilson.

In 1920 he joined the faculty of Bryn Mawr College where he remained until 1930. At this time he returned to Columbia University as a Professor of Zoology and again enjoyed association with Professor Wilson, then *Emeritus* in residence. He served the Department well as chairman but disliked administrative work. He became Da Costa Professor of Zoology, 1950–1959, and Da Costa Professor *Emeritus,* 1959–1962. On retiring from Columbia he joined the faculty of Duke University as Guest Professor. He died on March 22, 1962, in Durham, North Carolina.

Schrader was a member of the National Academy of Science. He served as Vice-President of Section F of the AAAS in 1947, and as President of the American Society of Zoologists in 1952. He was a Trustee of the Marine Biological Laboratory from

1934 to 1951 and became a Life Member in 1959. He was a member of several other scientific societies.

Schrader gave generously of his time and energy for editorial services; serving on the Editorial Boards of CHROMOSOMA, BIOLOGICAL BULLETIN, JOURNAL OF MORPHOLOGY, COLUMBIA BIOLOGICAL MONOGRAPHS, and the JOURNAL OF BIOCHEMICAL AND BIOPHYSICAL CYTOLOGY.

His bibliography indicates the nature, extent, and significance of his work. From 1920–1961 he had over 60 publications, including a monograph, *The Sex Chromosomes,* and a book on *Mitosis* published in 1944 with a second edition in 1953.

His early work dealt with the biology and pathology of fishes, followed by chromosome cytology related to sex determination and parthenogenesis. Next came studies of mitosis and its problems. Later work was characterized by the use of cytochemical and cytophotometric methods and their applications to cytological problems and to evolution. For most of his career he was preeminent in the field of chromosome cytology in many of its significant aspects. I am certain that Professor E. B. Wilson, after his retirement, was highly pleased to have Schrader as his successor at Columbia.

He and his wife, Sally Hughes Schrader, both first rate naturalists, and collectors and outstanding cytologists, made several trips to the tropics where they obtained material used in their cytological studies. He also found great joy in his collecting in many eastern and southern states.

It is fair to judge a scientist partly by his academic progeny and their contributions. I will not name them but Franz Schrader was entitled to feel a great gratification from this source.

Many of you were aware that for many years Schrader had health problems, which made it necessary for him to husband his energy. His accomplishments are the more remarkable in view of this.

He had many cultural and intellectual interests in music and literature. He was a collector of fine oriental rugs and a connoisseur of European wines. His lifetime interest in salt water fishing was fulfilled during recent summers at Pasque Island.

The warmth of his friendship and wisdom of his counsel were more appreciated than can easily be expressed in words.

2. The Staff

EMBRYOLOGY

I. Instructors

James D. Ebert, Director, Department of Embryology, Carnegie Institution of Washington, in charge of course
Allison L. Burnett, Associate Professor of Biology, Western Reserve University
Richard A. Cloney, Instructor in Zoology, University of Washington
Katsuma Dan, Professor of Biology, Tokyo Metropolitan University
Robert L. DeHaan, Staff Member, Department of Embryology, Carnegie Institution of Washington
A. A. Moscona, Professor of Zoology, University of Chicago
John W. Saunders, Jr., Professor of Biology, Marquette University

II. Laboratory Assistants

John M. Arnold, Department of Zoology, University of Minnesota
Sidney B. Simpson, Jr., Department of Zoology, Tulane University

PHYSIOLOGY

I. CONSULTANTS

MERKEL H. JACOBS, Professor of Physiology, University of Pennsylvania
ARTHUR K. PARPART, Professor of Biology, Princeton University
ALBERT SZENT-GYÖRGYI, Director, Institute for Muscle Research, Marine Biological Laboratory
W. D. MCELROY, Director, McCollum-Pratt Institute, Johns Hopkins University

II. INSTRUCTORS

J. WOODLAND HASTINGS, Associate Professor of Biochemistry, University of Illinois, in charge of course
PHILIP E. HARTMAN, Associate Professor of Biology, Johns Hopkins University
VINCENT MASSEY, Sheffield University
SHINYA INOUÉ, Professor of Cytology, Dartmouth College
K. E. VAN HOLDE, Associate Professor of Physical Chemistry, University of Illinois
JERARD HURWITZ, Associate Professor, New York University
H. O. HALVORSON, Associate Professor, University of Wisconsin

III. LABORATORY ASSISTANT

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BOTANY

I. CONSULTANT

WILLIAM RANDOLPH TAYLOR, Professor of Botany, University of Michigan

II. INSTRUCTORS

RICHARD C. STARR, Professor of Botany, Indiana University, in charge of course
BOHUSLAV FOTT, Professor of Botany, Charles University, Prague
WALTER R. HERNDON, Professor of Botany, University of Tennessee
ROBERT ROSS, British Museum (Natural History), London

III. LABORATORY ASSISTANTS

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MICHAEL WYNNE, Henry Shaw School of Botany, Washington University

INVERTEBRATE ZOOLOGY

I. CONSULTANTS

F. A. BROWN, JR., Morrison Professor of Biology, Northwestern University
LIBBIE H. HYMAN, American Museum of Natural History
ALFRED C. REDFIELD, Woods Hole Oceanographic Institution

II. INSTRUCTORS

CLARK P. READ, Professor of Biology, Rice University, in charge of course
JOHN M. ANDERSON, Professor of Zoology, Cornell University

James Case, Associate Professor of Zoology, State University of Iowa
G. F. Gwilliam, Associate Professor of Biology, Reed College
W. D. Russell Hunter, Lecturer in Zoology, University of Glasgow
Roger Milkman, Associate Professor of Zoology, Syracuse University
Richard C. Sanborn, Professor of Zoology, Purdue University

III. Assistants

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ECOLOGY

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B. H. Ketchum, Woods Hole Oceanographic Institution
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H. L. Sanders, Woods Hole Oceanographic Institution
R. L. Miller, Department of Biology, University of Saskatchewan
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Peter J. Wangersky, Bingham Oceanographic Laboratory, Yale University
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III. Laboratory Assistants

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Mrs. Deborah Lawrence Harlow, Librarian
Carl O. Schweidenback, Manager, Supply Department
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Robert Kahler, Superintendent, Buildings and Grounds
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Spector, Abraham, Instructor, Harvard Medical School, Howe Laboratories
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Spindel, William, Associate Professor, Rutgers University

Spirtes, Morris A., Associate Professor of Pharmacology, Hahnemann Medical College and Hospital
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Strittmatter, Philipp, Associate Professor of Biochemistry, Washington University
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Taylor, William Randolph, Professor of Botany, University of Michigan
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Torch, Reuben, Associate Professor of Zoology, University of Vermont
Trager, William, Associate Professor, Rockefeller Institute
Travis, David M., Assistant Professor of Pharmacology, University of Florida
Troll, Walter, Associate Professor, New York University School of Medicine
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Van Holde, K. E., Associate Professor, University of Illinois
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Villee, Claude A., Jr., Associate Professor of Biological Chemistry, Harvard University Medical School
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Warren, Leonard, Research Medical Officer, National Institutes of Health
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Wichterman, Ralph, Professor of Biology, Temple University
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Wilber, Charles G., Professor of Biological Sciences, Kent State University
Willey, Charles H., Professor of Biology, New York University
Wilson, Walter L., Associate Professor of Physiology, University of Vermont, College of Medicine
Winters, Robert W., Associate Professor of Pediatrics, College of Physicians and Surgeons
Zimmerman, Arthur M., Assistant Professor of Pharmacology, State University of New York, Downstate Medical Center
Zwilling, Edgar, Professor of Biology, Brandeis University

Lalor Fellows, 1962

Kamiya, N., Osaka University, Japan
Laufer, Hans, Johns Hopkins University
Nasatir, Maimon, Brown University
Winters, Robert W., College of Physicians and Surgeons

Lillie Fellow, 1962

MINGANTI, ANTONIO, University of Palermo, Italy

Grass Fellows, 1962

KRIEBEL, MAHLON E., University of Washington
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GOETZ, FREDERICK C., University of Minnesota
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Szent-Györgyi, Gyula, Institute for Muscle Research, Marine Biological Laboratory
Szent-Györgyi, Marta, Institute for Muscle Research, Marine Biological Laboratory
Tam, Sidney L., University of Chicago
Ulrich, Sara Ann, Chicago, Illinois
Veeder, Carolyn, Swarthmore College
Veeger, C., University of Amsterdam
Wallstrom, Natalie, Dartmouth Medical School
Wandel, Ted, Harvard University
Watters, Christopher D., Princeton University
Westhoff, Douglas, St. Louis University
Williams, Mary, Woods Hole
Wolfenden, Pamela, Harvard University
Wynne, Michael J., Washington University

Library Readers, 1962

Ball, Eric G., Professor of Biological Chemistry, Harvard Medical School
Berne, Robert M., Professor of Physiology, Western Reserve University School of Medicine
Bersohn, Richard, Associate Professor of Chemistry, Columbia University
Buck, John, Research Physiologist, National Institutes of Health
Butler, Elmer G., Professor of Biology, Princeton University
Carlson, Francis D., Professor and Chairman, Department of Biophysics, Johns Hopkins University
Chase, Aurin M., Professor of Biology, Princeton University
Clark, Arnold M., Professor of Biological Sciences, University of Delaware
Clifford, Sister Adele, Professor of Biology, College of Mount St. Joseph
Cohen, Seymour S., Professor of Biochemistry, University of Pennsylvania
Davis, Bernard D., Professor of Bacteriology, Harvard Medical School
Eder, Howard A., Professor of Medicine, Albert Einstein College of Medicine
Eisen, Herman N., Professor of Microbiology, Washington University School of Medicine
Fawcett, Don W., Hersey Professor of Anatomy, Harvard Medical School

Forer, Arthur, Graduate Student, Dartmouth Medical School
Fries, E. F. B., Professor, City College of New York
Gabriel, Mordecai L., Associate Professor of Biology, Brooklyn College
Gagne, Francois, Assistant of Pathology, Laval University
Ginsberg, Harold S., Professor of Microbiology, University of Pennsylvania
Glaser, Donald A., Visiting Professor of Biophysics, Massachusetts Institute of Technology
Green, James W., Professor of Physiology, Rutgers University
Green, Paul B., Associate Professor of Zoology, University of Pennsylvania
Gurewich, Vladimir, Clinical Associate Physician, New York Medical College
Handler, Philip, Professor of Biochemistry, Duke University
Hodes, Robert, Research Associate in Pediatrics, Mount Sinai Hospital
Isselbacher, Kurt J., Assistant Professor of Medicine, Harvard Medical School
Jacobs, M. H., Emeritus Professor, University of Pennsylvania
Karnovsky, Manfred L., Associate Professor of Biological Chemistry, Harvard Medical School
Karush, Fred, Professor of Microbiology, University of Pennsylvania
Klein, Morton, Professor, Temple University Medical School
Klotz, Irving M., Professor of Chemistry, Northwestern University
Lauffer, Max A., Professor of Biophysics, University of Pittsburgh
Levine, Rachmiel, Professor of Medicine, New York Medical College
Lineaweaver, Thomas H., Marine Biological Laboratory
Loewenfeld, Irene E., Instructor in Ophthalmology, Columbia University
McDonald, Sister Elizabeth Seton, Chairman, Dept. of Biology, College of Mount St. Joseph
Novikoff, Alex B., Research Professor, Albert Einstein College of Medicine
Petrie, Asenath, Research Associate in Psychiatry, Harvard University
Ray, Peter M., Associate Professor of Botany, University of Michigan
Roth, Jay S., Professor of Biochemistry, University of Connecticut
Rowland, Lewis P., Assistant Professor of Neurology, College of Physicians and Surgeons
Sager, Ruth, Research Associate, Columbia University
Scott, Allan, Professor of Biology, Colby College
Shemin, David, Professor of Biochemistry, Columbia University
Sinex, Francis M., Chairman, Biochemistry Department, Boston University School of Medicine
Stetten, DeWitt, Associate Director in charge of Research, National Institutes of Health
Stetten, Marjorie R., Chemist, National Institutes of Health
Steinhardt, Jacinto, Director, Operations Evaluation Group, Massachusetts Institute of Technology
Wainio, Walter, Professor of Biochemistry, Rutgers University
Warner, Robert C., Professor of Biochemistry, New York University School of Medicine
Weiss, Leon, Associate Professor of Anatomy, Johns Hopkins University School of Medicine
Wheeler, George E., Assistant Professor of Biology, Brooklyn College
Wilson, T. Hastings, Associate Professor of Physiology, Harvard Medical School
Yntema, Chester L., State University of New York, Syracuse
Zinn, Donald J., Chairman, Dept. of Zoology, University of Rhode Island
Zorzoli, Anita, Professor of Physiology, Vassar College

Students, 1962

All students listed completed formal course program, June 19 to July 28. Asterisk indicates students completing Post-Course research program, July 29 to September 1.

EMBRYOLOGY

Elizabeth E. Ballentine, Vassar College
*Gail Bottomly, University of North Carolina
*John J. Coffey, Johns Hopkins University
Michael F. Collins, Johns Hopkins University
*John F. Fallon, Marquette University
*Ira N. Feit, Princeton University
Caleb E. Finch, Rockefeller Institute

*Gary L. Freeman, University of Chicago
Ann Gold, Syracuse University
Victor Jaccarini, Woodstock College
*Shlomith Lerman, Radcliffe College
Steven W. Matthysse, Rockefeller Institute
*Ellen Mutterperl, Western Reserve University
Nancy R. Parker, University of Texas
*Judith H. Payne, Duke University
Carol Ann Ryder, University of Wisconsin
Jarid Simons, Yale University
Lewis D. Smith, Indiana University
Joan L. Thomson, Iowa State University
*Elaine S. Valliere, Catholic University

PHYSIOLOGY

Robert G. Cassens, University of Wisconsin
*Cornelia P. Channing, Radcliffe Graduate School
*Kwen-Sheng Chiang, Princeton University
*Lawrence B. Cohen, Columbia University
*Richard H. Colby, University of California
*Henry A. DePhillips, Jr., Northwestern University
Anatol Eberhard, Harvard University
*Milan Fiala, Massachusetts General Hospital
*Lincoln E. Ford, University of Rochester
*Paul German, Université Laval
*Donald A. Glaser, Massachusetts Institute of Technology
David L. Greenman, Purdue University
*Andrée Guindon, Université de Montreal
*Carol Kepler, University of North Carolina
Kenneth N. Krischer, University of Miami
*Samuel A. Latt, Harvard Medical School
*Gerhard Malnic, University of São Paulo
Manuel Ochoa, Jr., Columbia, College of Physicians and Surgeons
*Richard Pannbacker, Kansas State University
*Ronald E. Pearlman, Harvard University
William E. Robinson, Purdue University
*Judah L. Rosner, Yale University
*John R. Roth, Johns Hopkins University
Harriet Rouse, St. Louis University Medical School
*Alan A. Rozycki, Dartmouth Medical School
*Stasko, Aivars, University of Toronto
*Lila M. Williams, Yale University
Joseph Yourno, Johns Hopkins University

INVERTEBRATE ZOOLOGY

Hani K. Battikh, Iowa State University
*Stephen C. Brown, George Washington University
*Dorothea P. Caskey, University of Arizona
Laura Chunosoff, New York University
*Charles Daniels, Jr., Fairfield University
*Fred A. Diehl, Western Reserve University
Robert S. Eisenberg, Harvard University
Roger Dean Farley, State University of Iowa
James S. Farris, University of Massachusetts
James M. Freed, University of Illinois
Ann Gale, Rockefeller Institute
*Wm. Einar Gall, Hamilton College

*Helen T. Ghiradella, Cornell University
Howard L. Gillary, Johns Hopkins University
George C. Gorman, Cornell University
Faith H. Gray, Chatham College
Lewis Greenwald, Syracuse University
Carol Anne Grinager, Wilson College
*Charles D. Hardy, Cornell University
Edward F. Haskins, University of Minnesota
*John R. Kyrk, Syracuse University
Grace C. Lehman, Drew University
Jessie G. Levine, McMaster University
David McCoy, University of Iowa
Dorothy Merrill, University of Michigan
*D. Christine Nauman, University of Michigan
*Judith Newman, Dalhousie University
Kathleen Petersen, Syracuse University
Robert L. Philibert, University of Missouri
*Nancy K. Pianfetti, Purdue University
Sherwood Schearer, Lafayette College
Sister M. Rosarii Schmeer, University of Notre Dame
Gilbert Shibley, University of Oregon
*Jon G. Stanley, University of Missouri
Kathleen L. Terrell, University of Minnesota
Gayle R. Tryon, Cornell University
Raghunath Virkar, University of Minnesota
*Mary J. West, University of Michigan

BOTANY

*Richard H. Babcock, Brigham Young University
*Lois Benson, New York State Department of Health
*Richard Buggeln, Bucknell University
*Brother Joseph Cain, University of Texas
S. Chantanachat, University of Texas
*Joanne Dowell, Dalhousie University
*John H. Green, University of Louisville
Louis A. Hanic, University of British Columbia
*Daniel E. James, Indiana University
*Margaret Lloyd, Bryn Mawr College
*Rev. Alan Joseph McCarthy, Fordham University
William G. Merz, Drew University
Virginia M. Morzenti, University of Michigan
John B. Palmer, Oberlin College
*Russell Rhodes, University of Tennessee
Michael J. Schneider, University of Tennessee
Carolyn M. Silsby, University of Wisconsin
Joanne K. Solem, City College of New York
*Donald R. Tindall, University of Louisville
James R. Wheatley, Jr., East Carolina College

ECOLOGY

John H. Bushnell, Washington and Jefferson College
Jeremy Chapman, Montana State University
Eva A. Claus, New York University
Richard T. Cronin, Fordham University
Josephine Doherty, National Science Foundation
Rev. George L. Drury, Boston College
Brother J. M. Frenette, Seychelles College

Juan G. Gonzalez, University of Puerto Rico
Donald C. Gordon, Jr., Hamilton College
Ronald D. Harris, Dalhousie University
Laura Livingston, Goucher College
Emily K. Shirley, Vassar College
Paul E. Smith, State University of Iowa
Jon A. Sperling, Paul Smith's College
John H. Tietjen, University of Rhode Island

4. FELLOWSHIPS AND SCHOLARSHIPS, 1962

Bio Club Scholarship:
Joanne K. Solem, Botany Course
Gary N. Calkins Memorial Scholarship:
Stephen Brown, Invertebrate Zoology Course
Lucretia Crocker Scholarship:
Russell Rhodes, Botany Course
Edwin Linton Memorial Endowment of the Washington and Jefferson College:
John Bushnell, Ecology Course

5. Tabular View of Attendance, 1958–1962

	1958	*1959*	*1960*	*1961*	*1962*
Investigators—Total	410	427	458	456	494
Independent	203	215	231	224	235
Under Instruction	39	45	42	32	44
Library Readers	54	51	50	49	56
Research Assistants	114	116	135	151	159
Students—Total	138	134	122	130	121
Invertebrate Zoology	55	55	49	40	38
Embryology	22	23	20	21	20
Physiology	27	27	28	28	28
Botany	18	20	18	19	20
Ecology	16	15	13	22	15
Total Attendance	548	561	580	586	615
Less persons represented as both investigators and students	5	4	2	1	4
	543	557	578	585	611
Institutions Represented—Total	142	143	144	132	118
By Investigators	110	98	83	107	81
By Students	74	73	61	70	57
Schools and Academies Represented					
By Investigators	2	2	5	3	3
By Students		12	2		2
Foreign Institutions Represented	26	38	14	28	31
By Investigators	20	29	11	21	17
By Students	6	9	3	7	14

6. Institutions Represented 1962

Albert Einstein College of Medicine
American Museum of Natural History
American University
Amherst College
Massachusetts General Hospital
Massachusetts Institute of Technology
Massachusetts, University of
Miami, University of

Aquacultural Research Corporation
Argonne National Laboratory
Arizona, University of
Boston College
Brandeis University
Brooklyn College
Brown University
Brigham Young University
Bryn Mawr College
Bucknell University
Buffalo, University of
California Institute of Technology
California, University of
Carnegie Institution of Technology
Carnegie Institution of Washington
Catholic University of Washington
Chatham College
Chicago Medical School
Chicago, University of
Cincinnati, University of
City College of New York
Columbia University
Columbia University, College of Physicians & Surgeons
Cornell University
Dartmouth College
Drew University
Drexel Institute of Technology
Duke University
East Carolina College
Emory University
Fairfield University
Fels Research Institute
Florida State University
Fordham University
George Washington University
Goucher College
Hahnemann Medical School
Hamilton College
Harvard University
Harvard University Medical School
Illinois, University of
Indiana University
Institute for Muscle Research
Iowa State University
Jersey State College
Johns Hopkins University
Kansas University
Kansas State University
Kent State University
Lafayette College
Sarah Lawrence College
Louisville, University of
Loyola College
Marquette University
Maryland, University of
Massachusetts Eye and Ear Infirmary
Michigan, University of
Michigan State University Oakland
Minnesota, University of
Missouri, University of
Montana State University
Mount Holyoke College
New York State University of Long Island
New York State University at Brooklyn
New York State University Medical School at Syracuse
New York University, Bellevue Medical Center
New York University, School of Dentistry
New York University, Washington Square College
North Carolina State College
North Carolina, University of
Northwestern University
Notre Dame University
Oberlin College
Ohio State University
Oklahoma, University of
Oregon, University of
Pennsylvania, University of
Pennsylvania Medical School, University of
Pittsburgh, University of
Pomona College
Princeton University
Purdue University
Queens College
Radcliffe College
Reed College
Rhode Island, University of
Rochester, University of
Rockefeller Institute
Russell Sage College
Rutgers University
St. Louis University
Seton Hill College
Smith College
Stanford University
Syracuse University
Temple University
Tennessee, University of
Texas, University of
Tulane University
Vassar College
Vermont, University of
Veterans Administration Hospital
Virginia, University of
Washington and Jefferson College
Washington University Medical School
Washington University
Western Reserve University
Wilson College
Wisconsin, University of
Woodstock College
Yale University

Foreign Institutions Represented

Chulalongkorn University, Bangkok, Thailand
University of British Columbia, Vancouver, B. C.
Université Laval, Quebec
Université de Montreal, Quebec
University of São Paulo, Brazil
University of Alberta, Calgary, Alberta
University of Toronto, Ontario
Seychelles College, Seychelles Island, India
University of Puerto Rico, Puerto Rico
The Hebrew University, Jerusalem, Israel
University of Queensland, Queensland, Australia
Embassy of the Syrian Arab Republic, Syria
McMaster University, Hamilton, Ontario
Dalhousie University, Halifax, Nova Scotia
Cambridge University, Cambridge, England
Edinburgh University, Edinburgh, Scotland
Tokyo Metropolitan University, Tokyo, Japan
Charles University, Prague
University of Glasgow, Scotland
Osaka University, Japan
University of Sheffield, England
Inst. de Biologia, Mexico
University of Saskatchewan, Saskatoon, Saskatchewan
University of Palermo, Italy
Birmingham University, England
British Museum, London, England
University of Pisa, Italy
Hokkaido University, Japan
University of Ljubjana, Yugoslavia
State University, Torino, Italy
Fukushima Medical College, Japan

Supporting Institutions, Agencies and Individuals

Abbott Laboratories
Associates of the Marine Biological Laboratory
Atomic Energy Commission
The Burroughs Wellcome Fund
CIBA Pharmaceutical Products, Inc.
Josephine B. Crane Foundation
Dr. William D. Curtis
Eli Lilly and Company
The Grass Foundation
Hoffman-LaRoche Inc.
The Lalor Foundation
The Merck Company Foundation
National Institutes of Health
National Science Foundation
Office of Naval Research
Olin Mathieson Chemical Corporation, Charitable Trust
The Rockefeller Foundation
Sandoz Pharmaceutical Inc.
Gerard Swope, Jr.
Mr. and Mrs. John Swope
The Upjohn Company
Wallace Laboratories
James Wickersham

7. Friday Evening Lectures, 1962

July 6	Alan Hodgkin	Conduction of impulses in nerve and muscle. I
July 9	Alan Hodgkin	Conduction of impulses in nerve and muscle. II
July 13	Mac V. Edds, Jr.	Intercellular matrices as models for developmental analysis
July 20	Shields Warren	Radiobiologic research and its role in radiation safety
July 27	Carroll M. Williams	Insect metamorphosis and the management of genetic information
August 3	Noburo Kamiya	Protoplasmic streaming

August 10	George W. Beadle	Three-letter words in a four-letter language
August 17	Sir Lindor Brown	Memorial lecture to Otto Loewi
August 24	Eduardo de Robertis	Ultrastructure and chemical organization of isolated nerve endings

8. Tuesday Evening Seminars, 1962

July 3	Peter Rieser	Amino acid transport in the human red cell: kinetics and mechanism
	Roberts Rugh	Some embryonic effects of ionizing radiations
	Ernst Scharrer	Electron microscopy of neurosecretory cells in the preoptic nucleus of the toadfish (*Opsanus tau*)
July 10	Roger Milkman	A common mechanism for temperature adaptation and crossvein deformation in *Drosophila*
	Philip B. Dunham	Adaptation of *Tetrahymena* to a high NaCl environment: A further report
	C. C. Speidel and R. H. Cheney	Time-lapse motion pictures of zygote versus gamete irradiation (ultraviolet and x-ray) in *Arbacia*
July 17	John B. Morrill and Elaine N. Dow	Organ and ontogenetic patterns of multiple forms of hydrolytic enzymes in *Limnea palustris*
	Herman W. Lewis	Genetic control and regulation of dopa oxidase in *Drosophila melanogaster*
	G. W. deVillafranca	The A and I bands in contracting *Limulus* muscle
July 24	Alberto Monroy and M. L. Vittorelli	On the utilization of ^{14}C from glucose for amino acids and protein synthesis in the sea urchin embryo
	DeWitt Stetten, Jr.	Reversible enzymatic reduction of insulin
	Alex B. Novikoff	Golgi apparatus and lysosomes in vertebrate neurons
July 31	Albert Szent-Györgyi	Chemistry of the thymus gland
	Andrew Hegyeli	Chemistry of the thymus gland
	Ben Kaminer	Water and contraction of glycerol-extracted muscle
August 7	Reuben Torch	Regeneration studies on a brackish-water ciliate, *Tracheloraphis* sp.
	Abraham Spector	Lens amino peptidase
	Paul R. Gross	"Messenger" RNA and the cell cycle in a fission yeast
	Leonard Nelson	"Actin" localization in sperm

August 14	M. A. Spirtes	An effect of chlorpromazine on a sub-cellular and a cellular membrane
	M. Rockstein	Alpha glycerophosphate dehydrogenase in the flight muscle of insects and its possible role in the aging of flight ability
	M. Lauffer	Water and the substructure of a virus
August 21	A. J. deLorenzo	Ultrastructure of nerve fibers and synaptic junctions in gustatory receptors
	George Wald	Duplex vision in the crayfish
	D. Watkins, S. J. Cooperstein and A. Lazarow	Studies on the mechanism by which alloxan alters the permeability of islet cell membranes to mannitol

9. Members of the Corporation, 1962

Life Members

Brodie, Mr. Donald M., 522 Fifth Avenue, New York 18, New York
Cole, Dr. Elbert C., 2 Chipman Park, Middlebury, Vermont
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Payne, Dr. Fernandus, Indiana University, Bloomington, Indiana
Plough, Dr. H. H., Amherst College, Amherst, Massachusetts
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Scott, Dr. Ernest L., Columbia University, New York City, New York
Schrader, Dr. Sally, Duke University, Durham, North Carolina
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Warren, Dr. Herbert S., 2768 Egypt Road, Audubon, Pennsylvania

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Albert, Dr. Alexander, Mayo Clinic, Rochester, Minnesota

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Ballard, Dr. William W., Department of Zoology, Dartmouth College, Hanover, New Hampshire

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Baylor, Dr. Martha B., Marine Biological Laboratory, Woods Hole, Massachusetts

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Cornman, Dr. Ivor, Hazleton Laboratories, Box 333, Falls Church, Virginia

Costello, Dr. Donald P., Department of Zoology, University of North Carolina, Chapel Hill, North Carolina

Costello, Dr. Helen Miller, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina

Crane, Mr. John O., Woods Hole, Massachusetts

Crane, Dr. Robert K., Department of Biochemistry, Chicago Medical School, Chicago 12, Illinois

Croasdale, Dr. Hannah T., Dartmouth College, Hanover, New Hampshire

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Sturtevant, Dr. Alfred H., California Institute of Technology, Pasadena 4, California

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Sulkin, Dr. S. Edward, Department of Bacteriology, University of Texas, Southwestern Medical School, Dallas, Texas

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Tasaki, Dr. Ichiji, Laboratory of Neurophysiology, NINDB, Bethesda 14, Maryland

Tashiro, Dr. Shiro, University of Cincinnati Medical College, Cincinnati, Ohio

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Taylor, Dr. Wm. Randolph, Department of Botany, University of Michigan, Ann Arbor, Michigan

Taylor, Dr. W. Rowland, Department of Oceanography, Johns Hopkins University, Baltimore 18, Maryland

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Trinkaus, Dr. J. Philip, Department of Zoology, Osborn Zoological Laboratories, Yale University, New Haven, Connecticut

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Tweedell, Dr. Kenyon S., Department of Biology, University of Notre Dame, Notre Dame, Indiana

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Vincent, Dr. Walter S., Department of Anatomy, University of Pittsburgh, Pittsburgh 13, Pennsylvania

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Whiting, Dr. Phineas, Department of Zoology, University of Pennsylvania, Philadelphia 4, Pennsylvania

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Willier, Dr. B. H., Department of Biology, Johns Hopkins University, Baltimore 18, Maryland

Wilson, Dr. J. Walter, Department of Biology, Brown University, Providence 12, Rhode Island

Wilson, Dr. Walter L., Department of Physiology, University of Vermont College of Medicine, Burlington, Vermont

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Zirkle, Dr. Raymond E., Department of Radiobiology, University of Chicago, Chicago 37, Illinois

Zorzoli, Dr. Anita, Department of Physiology, Vassar College, Poughkeepsie, New York

Zweifach, Dr. Benjamin, New York University Medical Center, New York 16, New York

Zwilling, Dr. Edgar, Department of Biology, Brandeis University, Waltham 54, Massachusetts

Associate Members

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Alton, Mrs. Benjamin
Andres, Mrs. William
Armstrong, Mrs. Philip B.
Bacon, Mr. and Mrs. Robert
Bacon, Dr. Catherine L.
Ball, Mrs. Eric G.
Barbour, Mr. and Mrs. Lucius H.
Barker, Mr. and Mrs. James R.
Barrows, Mrs. Albert
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Calkins, Mrs. Gary N.
Calkins, Mr. and Mrs. G. Nathan, Jr.
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Gifford, Mr. and Mrs. John A.
Gifford, Mr. and Mrs. Prosser
Gifford, Mrs. William
Gilchrist, Mr. and Mrs. John M.
Gildea, Dr. and Mrs. E. F.
Green, Miss Gladys M.
Greer, Mrs. Margaret Jewett
Greer, Mr. and Mrs. William Hershey, Jr.
Gulesian, Mrs. Paul J.
Haigh, Mrs. R. H.
Hamlen, Mr. and Mrs. J. Monroe
Harrell, Mr. and Mrs. Joel E.
Harrington, Mr. and Mrs. Robert D.
Harvey, Dr. Ethel Browne
Harvey, Dr. and Mrs. Richard
Harrington, Mrs. A. W.
Hervey, Mrs. John P.
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Lobb, Mr. and Mrs. John
Loeb, Dr. and Mrs. Robert F.
Marsland, Dr. and Mrs. Douglas A.
Marvin, Mrs. Walter Taylor
Mast, Mrs. S. O.
Mather, Mr. Frank J., III
McCusker, Mrs. Paul T.
McElroy, Dr. and Mrs. W. D.
McKelvy, Mr. John E.
Meigs, Dr. and Mrs. J. Wister
Mitchell, Mrs. James McC.
Mitchell, Mrs. Philip
Mixter, Mrs. William Jason
Mosser, Mrs. Benjamin D.
Motley, Mrs. Thomas
Newton, Miss Helen K.
Nichols, Mrs. George
Nims, Mrs. E. D.
The Aaron E. Norman Fund, Inc.
Packard, Mrs. Charles

Park, Mr. and Mrs. Malcolm S.
Pennington, Miss Anne H.
Philippe, Mr. Pierre
Putnam, Mr. William A., III
Redfield, Dr. and Mrs. Alfred C.
Reznikoff, Dr. and Mrs. Paul
Riggs, Mr. Lawrason
Rivinus, Mrs. F. M.
Robinson, Dr. Miles
Root, Dr. and Mrs. Walter S.
Rudd, Mr. and Mrs. H. W. Dwight
Sands, Miss Adelaide G.
Saunders, Mr. and Mrs. Lawrence
Shiverick, Mrs. Arthur
Sinclair, Mr. and Mrs. W. Richardson
Speidel, Mrs. Carl C.
Stone, Mr. and Mrs. Leo
Stone, Mrs. Samuel M.
Straus, Mr. and Mrs. Donald B.
Stunkard, Mrs. Horace
Swope, Mr. David
Swope, Mr. and Mrs. Gerard, Jr.
Swope, Miss Henrietta H.
Szent-Györgyi, Dr. and Mrs. Albert
Tompkins, Mr. and Mrs. B. A.
Ware, Mrs. J. Leslie
Webster, Mrs. Edwin S.
Whiteley, Miss Mabel W.
Wickersham, Mrs. James H.
Wilhelm, Dr. and Mrs. Hilmer J.
Williston, Mr. Samuel
Wilson, Mrs. Edmund B.
Wolfinsohn, Mrs. W.

New in 1962

Forbes, Dr. Alexander
Garfield, Miss Eleanor
Gillette, Mr. and Mrs. Robert S.
McLane, Mrs. Huntington
McVitty, Mrs. A. E.
Minis, Mr. and Mrs. Abram J., Jr.
Makrauer, Mr. S. Lang
Stone, Dr. William, Jr.

V. REPORT OF THE INSTRUCTION COMMITTEE ON EDUCATIONAL POLICY

A year ago, in connection with certain questions raised in the Trustees' Meeting, the Instruction Committee was asked to re-examine the whole teaching policy at the MBL. In complying with this directive, the Committee views its role as primarily that of reporting on the present state of our courses in relation to various questions of quality of appropriateness that have been raised, rather than that of recommending a set of standards.

The Committee has not given undue weight to tradition, feeling that even founding fathers can hardly be expected to devise policy that will remain continuously appropriate with the passage of time. Nevertheless it may be of interest to summarize some past opinions on instruction policy. Paraphrasing from several of Director C. O. Whitman's reports in the early years of the century, from Director F. R. Lillie's report of 1921 (*Biol. Bull.*, **42**: 304–309, 1922), from the report of E. G. Conklin's nine-man Policy Committee of 1938 (*Biol. Bull.*, **77**: 15–27, 1939), and from Lillie's book of 1944, we find the following as rather consistently recurring principles, policies and implications:

1. That all policies of the Laboratory are subservient to the research progress of the individual investigators.

2. That instruction at the MBL must go hand in hand with research because "it is the business of the Laboratory to produce investigators as well as investigation." In agreement with this principle it is stated that (a) admission preference

should be given to students heading for professional careers in biology, and (b) the courses need not all be at the same level, some being more appropriate as basic background, and others as an introduction to research.

3. That subjects of courses should be fundamental and general rather than systematic and more special. We take this to mean that instruction should be confined to major subdivisions of the field of Biology.

4. Courses must be offered here to greater advantage than elsewhere, not as duplication of college or university courses. The use of marine material is several times mentioned in connection with the uniqueness of MBL courses, though never in connection with research at the MBL.

5. Instruction can be overdone. There should be a strict limit on the number of students and on the courses (traditionally four or five).

An additional general policy is of indirect significance, namely, that the Laboratory has no set program of research, but aims only to be in the forefront of the advance of biological sciences as represented in the research interests of the investigators. Implicit in this, we believe, are the expectations (a) that the courses be kept up-to-date as regards research progress in particular fields, and (b) that it is possible that the emphasis or orientation of a particular course could undergo even a major shift in the course of time.

One other point of historical interest: Between the lines of the 1921 report we perhaps detect evidence that the chemical Young Turks and the biological Old Guard were even then squaring off, for we read, "The fundamental problems trace back to the cell and are based ultimately on physics and chemistry; they thus require some of the special equipment of both of these fields."

Since the present Committee has no quarrel with any of the statements just reviewed, we turn next to the question of how our present courses measure up to these possible criteria:

1. "An MBL course should be in some sense unique." We have three comments:

(a) A very important but easily overlooked fact is that all MBL courses have been, are and will be unique in that they give the students an opportunity to be part of a unique research establishment and bring them in contact with a unique aggregation of research scientists.

(b) Not all courses need achieve uniqueness in the same way. The Zoology and Botany courses properly emphasize the opportunity to study living marine organisms in a research environment. The Physiology Course from its inception, and the Embryology Course in recent years, have derived their prominence more from the opportunity they offer to work simultaneously under several first-line specialists in currently active research fields.

(c) The preeminence of our Zoology Course over the years has given it a special kind of uniqueness in that as the study of invertebrates has been crowded out of college curricula, our course has become an accepted part of the training of biology majors in certain schools.

2. "An MBL course shall have as its primary objective the training of research scientists." We have two comments:

(a) We do not feel that this principle should be so rigid as necessarily to exclude the occasional exceptionally talented teacher who is prepared to take full advantage of an MBL course.

(b) We do not feel that all courses need be taught at the research level. We feel that a comprehensive basic background is the most important contribution that can be made by our courses in Zoology and Botany, whereas in Physiology, Ecology and Embryology a more eclectic approach, involving concentration on a few frontiers in modern science and the acquisition of specialized technical skills, may be the primary objective.

3. "Use marine material." We have two comments:

(a) We feel that the MBL has a responsibility in furthering the acquisition of knowledge in marine biology, not because this has been the Laboratory's tradition, or because of the edict of some past committee, but because there is a great need in this field and because we are uniquely equipped to serve it.

(b) On the other hand, a too rigid adherence to this principle would bring us into direct conflict with the even more fundamental policy of following where research leads us. Our Embryology course may in fact exemplify just such a change in emphasis in that its original concentration on studies of marine eggs and embryos—cleavage, cell lineage, morphogenesis—was a happy wedding of the appropriate use of our local material and interest in investigations that were then in the forefront of current embryological research, whereas present-day interest has shifted to other materials and problems. In other words, we should be satisfied, if it is necessary to preserve the emphasis of topical research, if marine material is brought into the Laboratory work wherever it is possible to use it to advantage, and into lecture in proportion to its background importance and its use in current research.

4. Finally, we append the following miscellaneous observations:

(a) We reaffirm the principle that content and conduct of all courses shall be the exclusive responsibility of the teaching staff and that neither Instruction Committee, Trustees nor Director should attempt to influence a course directly except at the time a new head is selected.

(b) In spite of principles, it may be necessary to compromise. For example, in spite of the apparent *a priori* desirability of staffing the Zoology course with specialists in marine invertebrates, it is in practice impossible—and has apparently always been so—if we insist also on research distinction in experimental science.

(c) It seems worthwhile to list criteria which we feel are *not* relevant in judging MBL courses. First, the popularity of the lectures. Many things contribute to this phenomenon, none of them crucial to whether a course is proper to be taught at the MBL. It would be quite possible to jam the auditorium with bird watchers if a bang-up course in ornithology were given here for several years.

A second meretricious criterion is student adjustment. It is common experience that students like to turn knobs and do not like to look through the microscope but this ought not to influence the orientation of a course into undue emphasis on experimentation at the expense of the more demanding discipline of meticulous observation and the acquisition of a firm foundation of basic background information.

In general summary, then, we feel that the quality of MBL instruction is good and the orientation of the courses satisfactory. We feel that it is more important to have courses of which we can be proud than one that "covers" this or that specific aspect of biology. It is, in fact, impossible to define a field of biology rigorously, or for a course to encompass all its aspects, and there has always been at the MBL a wide spectrum of opinion as to what should be taught, if anything, how it should be taught and who should teach it. We feel that these signs of discontent are healthy, and that as long as the same course can, for example, be criticized simultaneously by different individuals for containing too little and too much physiology, the Laboratory is not making serious mistakes.

Now, we want to turn briefly to one other matter rather ambiguously related to instruction, namely, Training Grants. Insofar as such programs have been incorporated into our Physiology, Embryology, Zoology and Ecology courses they appear to be having a clearly beneficial effect, certainly so far as physical facilities go, and it is hoped that the day does not come when we have to face the question of whether we could still run courses under our own steam. However, money being the root of all evil, the Committee urges particular caution in assuming such obligations in the future, lest an imbalance between instruction and individual research at the MBL be created. All proposals involving an increase in the number of students, increase in the number of courses, the setting up of specialized training courses, etc., should be scrutinized carefully with the following points in mind:

1. Does the proposal hamper in any way the investigative activity of the Laboratory in relation to space, equipment, facilities, personnel or living quarters?
2. Does the proposal really advance the science of biology in any fundamental sense or is it training a particular technique or method?

John B. Buck, *Chairman*
A. Lazarow
T. Hayashi
B. Ketchum
J. W. Green

VI. REPORT OF THE LIBRARIAN

In 1962, the Library received 1790 current journals, 57 new titles having been added during the year. Of the total, the Marine Biological Laboratory subscribed to 554, received 654 in exchange for the *Biological Bulletin* and 193 as gifts. The Woods Hole Oceanographic Institution subscribed to 123, received 205 in exchange and 61 as gifts.

The Laboratory purchased 98 books, received 107 complimentary copies (15 from the authors and 92 from the publishers) and accepted 34 miscellaneous gifts. The Institution purchased 42 books and received 10 gifts. The number of books accessioned totalled 291.

Through purchase, exchange and gift the Laboratory completed 16 journal sets and partially completed 18. The Institution completed three sets and partially completed four. A total of 4648 reprints were added to the collection, of which 2817 were of current issue.

The Library now contains 80,257 bound volumes, 950 having been sent to the bindery in 1962. The reprint collection numbers 223,700.

The photocopying machine has enabled the Library to lower the number of volumes sent out on interlibrary loan. There were 278 volumes sent out in 1962 compared to 553 in 1961, and 106 were borrowed from other libraries.

Acknowledgment is herewith made to Drs. W. D. Russell Hunter, J. A. Hartmann, Roberts Rugh and to the estate of Dr. Otto Loewi for presentations made during the year.

The Montgomery Memorial Fund has now been totally expended and it has enabled the Library to purchase 55 books and two journal sets which have greatly enriched the zoological literature.

The Falmouth Hospital plans on subscribing to certain medical journals which are not now in this Library, and permission has been given to store them here for reference. Thus, each year there is a decided increase in the use of our facilities.

Respectfully submitted,
Deborah L. Harlow,
Librarian

VII. REPORT OF THE TREASURER

The market value of the General Endowment Fund and the Library Fund at December 31, 1962, amounted to $1,994,709 as against book value of $1,242,771. This compares with values of $2,025,139 and $1,193,853, respectively, at the end of the preceding year. The average yield on the securities was 3.75% of the market value and 6.01% of book value. The total uninvested principal cash in the above accounts as of December 31, 1962 was $641.03. Classification of the Securities held in the Endowment Funds appears in the Auditor's report.

The market value of the pooled securities as of December 31, 1962 was $353,243 with uninvested principal cash of $818.83, the market value at December 31, 1961 being $373,641. The book value of the securities in this account was $302,453 on December 31, 1962, compared with $293,068 a year earlier. The average yield on market value was 3.53% and 4.13% of book value.

The proportionate interest in the Pool Fund Account of the various Funds as of December 31, 1962 is as follows:

Pension Funds	28.462%
General Laboratory Investment	50.515
Other:	
Bio Club Scholarship Fund	1.447
Rev. Arsenius Boyer Scholarship Fund	1.771
Gary N. Calkins Fund	1.657
Allen R. Memhard Fund	.321
F. R. Lillie Memorial Fund	5.583
Lucretia Crocker Fund	6.044
E. G. Conklin Fund	1.022
Jewett Memorial Fund	.538
M. H. Jacobs Scholarship Fund	.729
Anonymous Gift	1.911

Donations from the MBL Associates for 1962 were $5,305.00 as compared with $4,330 for 1961. Unrestricted gifts from foundations, societies and companies amounted to $34,519.

During the year we administrated the following Grants:

Investigators	*Training*	*MBL—Institutional*
8—NIH	6—NIH	2—NIH
5—NSF	1—NSF	3—NSF
1—Ford		2—ONR
		1—AEC
14	7	8

The amounts of grants vary in accordance with the investigator's project of research. An amount of 15% and 20%, based on amount expended, is allowed the Laboratory as overhead, which amounted to $43,405.

The Lillie Fellowship Fund with a market value of $98,498 and a book value of $92,400 as well as the investment in the General Biological Supply House with a book value of $12,700, is carried in the Balance Sheet, item "Other Investments." The General Biological Supply House fiscal year ended June 30, 1962, and had a profit after taxes of $302,657 as compared to $302,851 in 1961 and $314,034 in 1960 and $303,300 in 1959 and $218,210 in 1958. In the fiscal year 1962, the Marine Biological Laboratory received dividends from the General Biological Supply House of $38,100 as against $33,020 in 1961 and $30,480 in 1960 and $30,480 in 1959.

Following is a statement of the auditors:

To the Trustees of the Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheets of the Marine Biological Laboratory as of December 31, 1962 and 1961, the related statements of operation expenditures, income and current fund for the years then ended, and statement of funds for the year ended December 31, 1962. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the account records and such other auditing procedures as we considered necessary in the circumstances.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of the Marine Biological Laboratory at December 31, 1962, and the results of its operations for the year then ended on a consistent basis.

Boston, Massachusetts
March 18, 1963

Lybrand, Ross Bros. & Montgomery

James H. Wickersham,
Treasurer

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1962 and 1961

Investments

	1962	*1961*
Investments held by Trustee:		
Securities, at cost (approximate market quotation 1962—$1,995,000)	$1,242,771	$1,193,853
Cash	641	1,932
	1,243,412	1,195,785
Investments of other endowment and unrestricted funds:		
Pooled investments, at cost (approximate market quotation, 1962—$353,000) less $5,728 temporary investment of current fund cash	296,725	287,340
Other investments	139,677	138,546
Cash	15,135	12,764
Accounts receivable	31	41
	$1,694,980	$1,634,476

Plant assets

Land, buildings, Library and equipment (note)	4,900,749	4,795,960
Less allowance for depreciation (note)	1,247,149	1,189,121
	3,653,600	3,606,839
Construction in progress		105
U. S. Government obligations, at cost:		
$50,000 Treasury certificates, due 5/15/62		50,000
	$3,653,600	$3,656,944

Current Assets

Cash	118,732	65,623
Temporary investment in pooled securities	5,728	5,728
U. S. Treasury bills, due prior to 2/15/63, at cost	248,253	
Accounts receivable (U. S. Government, 1962—$41,579; 1961—$20,129	78,099	49,290
Inventories of specimens and Bulletins	41,903	43,712
Prepaid insurance and other	19,006	6,870
	$ 511,721	$ 171,223

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1962 and 1961

Endowment Funds

	1962	*1961*
Endowment funds given in trust for benefit of the Marine Biological Laboratory	$1,243,412	$1,195,785
Endowment funds for awards and scholarships:		
Principal	126,302	126,302
Unexpended income	11,353	9,600
	137,655	135,902
Unrestricted funds functioning as endowment	206,378	206,378
Retirement fund	94,048	81,790
Pooled investments—accumulated gain	13,487	14,621
	$1,694,980	$1,634,476

Plant Liability and Funds

Funds expended for plant, less retirements	4,900,749	4,796,065
Less allowance for depreciation charged thereto	1,247,149	1,189,121
	3,653,600	3,606,944
Unexpended plant funds		50,000
		3,656,944
	$3,653,600	$3,656,944

Current Liabilities and Funds

Accounts payable	43,253	30,337
Unexpended grants—research	344,437	52,837
Unexpended balances of gifts for designated purposes	9,461	8,878
Current fund	114,570	79,171
	$ 511,721	$ 171,223

Note—The Laboratory has since January 1, 1916 provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original costs of the assets.

MARINE BIOLOGICAL LABORATORY

Statements of Operating Expenditures, Income and Current Fund

Years Ended December 31, 1962 and 1961

Operating Expenditures

	1962	*1961*
Research and accessory services	$ 237,580	$ 192,973
Instruction	142,394	158,780
Library and publications (including book purchases—1962, $24,287; 1961, $20,561	72,514	67,189
Direct costs on research grants	378,163	177,938
Direct costs on institution support grants	98,043	75,220
	928,694	672,100
Administration and general	92,124	83,366
Plant operation and maintenance	102,632	118,993
Dormitories and dining	155,234	160,838
Additions to plant from current income	22,088	27,210
	1,300,772	1,062,507
Less depreciation included in plant operation and dormitories and dining above but charged to plant funds	65,061	48,419
	1,235,711	1,014,088

Income

	1962	*1961*
Research fees	99,314	102,081
Accessory services (including sales of biological specimens—1962, $29,898; 1961, $43,045	90,247	86,790
Instruction fees	27,439	27,730
Library fees, Bulletins, subscriptions and other	41,314	39,575
Dormitories and dining income	125,176	123,231
Grants for support of institutional activities:		
Instruction and training	127,988	148,078
Support Services	98,043	14,420
General	79,500	99,750
Reimbursements and allowances for indirect costs on specific research grants	421,568	195,716
Gifts used for current expenses	39,824	40,468
Investment income used for current expenses	120,697	110,333
Total current income	1,271,110	988,172
Excess current income (operating expenditures)	35,399	(25,916)
Current fund balance January 1	79,171	105,087
Current fund balance December 31	$ 114,570	$ 79,171

MARINE BIOLOGICAL LABORATORY

STATEMENT OF FUNDS

Year Ended December 31, 1962

	Balance Jan. 1, 1962	*Gifts & Other Receipts*	*Investment Income*	*Used for Current Expenses*	*Other Expenditures*	*Balance Dec. 31 1962*
Invested funds	$1,634,476	$ 66,633	$128,216	$119,710	$14,635	$1,694,980
Unexpended plant funds	$ 50,000	23,803	749		74,552	
Unexpended research grants	$ 52,837	1,025,149		733,549		$ 344,437
Unexpended gifts for designated purposes	$ 8,878	43,838		39,824	3,431	$ 9,461
Current fund	$ 79,171	35,399(1)				$ 114,570
		$1,194,822	$128,965	$893,083	$92,618	
Gifts		62,301				
Grants for research, training and support		1,013,575				
Net gain on sales of securities		46,493				
Appropriated from current income and other		37,054				
(1) Excess current income over expenditures		35,399				
		$1,194,822				
Expended for construction of new building and houses					74,522	
Scholarship awards					3,538	
Payments to pensioners					11,097	
Other					3,431	
					$92,618	

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1962

Securities held by Trustee:	*Cost*	*% of Total*	*Market Quotations*	*% of Total*	*Investment Income 1962*
General endowment fund:					
U. S. Government securities	$ 35,055	3.4	$ 35,536	2.1	$ 1,652
Corporate bonds	537,808	51.8	531,566	32.0	21,814
Preferred stocks	118,027	11.4	112,125	6.7	4,149
Common stocks	346,612	33.4	985,279	59.2	35,038
	1,037,502	100.0	1,664,506	100.0	62,653
General Educational Board endowment fund:					
U. S. Government securities	31,020	15.1	31,475	9.5	1,491
Other bonds	76,769	37.4	77,688	23.5	3,540
Preferred stocks	37,245	18.1	36,820	11.2	1,385
Common stocks	60,235	29.4	184,220	55.8	5,683
	205,269	100.0	330,203	100.0	12,099
Total securities held by Trustee	$1,242,771		$1,994,709		$ 74,752
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government securities	22,465	7.4	22,455	6.3	508
Corporate bonds	133,611	44.2	135,812	38.5	6,178
Common stocks	146,377	48.4	194,976	55.2	5,794
	302,453	100.0	353,243	100.0	12,480
Other investments:					
U. S. Government securities	7,000				350
Other bonds	47,842				1,999
Preferred stocks	3,728				130
Common stocks	46,530				39,369
Real estate	34,577				
	139,677				41,848
Total investments of other endowment and unrestricted funds	$ 442,130				$ 54,328
Total investment income					129,080
Custodian's fees charged thereto					(631)
Income of current funds temporarily invested in pooled securities					(233)
Investment income distributed to funds					$128,216
Current investment:					
U. S. Treasury bills, due prior to 2/15/63	$ 248,253		$ 250,000		$ 754

EFFECT OF THIOUREA ON MOULTING AND PUPATION OF THE SILKWORM, BOMBYX MORI L.

WANDA CHMURZYŃSKA AND LECH WOJTCZAK

Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw, Poland [1]

Insect blood, integument and other tissues contain the enzyme called phenoloxidase, or tyrosinase, which catalyzes the oxidation of various phenolic compounds to the corresponding quinones (for references see Sussman, 1949). Although there is much uncertainty about the role of phenoloxidase in the respiratory metabolism of insects, the function of phenoloxidase in the formation of insect cuticle is much better understood (*cf.* Mason, 1955; Hackman, 1958). Quinones, which are formed by the enzyme from phenols present in the cuticle, combine with proteins, thus forming the hard dark-colored layer of the cuticle. This process, called sclerotization and pigmentation, has been often investigated (Wigglesworth, 1947, 1948; Kawase, 1956). The view that phenoloxidase participates in the formation of insect cuticle is supported by numerous observations (Ito, 1953, 1954; Karlson, 1958; Kawase, 1960; Wojtczak, 1956), which show that the activity of the enzyme considerably increases during pupation and moulting, *i.e.*, at periods of intense cuticle-formation.

It seems thus interesting to investigate whether *in vivo* inhibition of phenoloxidase activity may affect moulting and pupation. Such attempts were first made by Dewitz (1901, 1902), who found that anoxia suppressed the hardening and the pigmentation of pupal cuticle of *Pieris brassicae*, and inhibited the pupation of *Lucilia caesar*. However, this effect, being rather unspecific, cannot be interpreted in terms of inhibition of the phenoloxidase alone. A more specific study on the inhibition of phenoloxidase was carried out by Fukuda (1953). He found that feeding silkworm larvae, *Bambyx mori* L., with mulberry leaves sprayed with thiourea produced serious disturbances both in larval moulting and in pupation.

We obtained similar results with a series of phenoloxidase inhibitors, including thiourea, injected into larvae of the waxmoth, *Galleria mellonella* L., and the silkworm, *Bombyx mori* L. (preliminary note: Wojtczak, 1954). The aim of the present investigation was to obtain more detailed information on the effects produced by an *in vivo* inhibition of phenoloxidase in silkworm larvae.

MATERIAL AND METHODS

Larvae of a yellow strain of the silkworm, *Bombyx mori* L., obtained from the Institute of Sericulture in Milanówek, were used.

From 5 to 10 μmoles of thiourea per gram body weight were introduced into the larvae by a microsyringe in the form of 0.2 *M* water solution. The total volume of the injected fluid was between 25 to 50 μl. per gram body weight. Control larvae were injected with the same volumes of 0.1 *M* KCl. In order to reduce bleeding the larvae were chilled before the injection and the wounds were sealed with paraffin.

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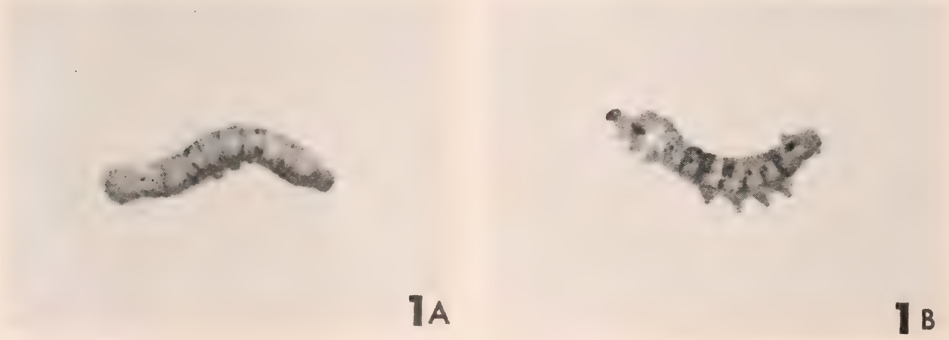
The activity of phenoloxidase was measured, with catechol as the substrate, in homogenates of whole insects by the procedure described earlier (Wojtczak, 1956).

For histological examination fragments of the integument were taken from the mid-dorsal part of the larvae and pupae, fixed in Bouin's fixative, sealed in paraffin, cut on a microtome and stained with pyronine and methyl green, or with silver nitrate.

RESULTS

Injection of as much as 10 μmoles thiourea per gram body weight had no immediate effect on the behavior of the larvae; they continued to feed or they spun normal cocoons. If the injection was made in the middle of the fourth or fifth instar, the larvae underwent normal larval moulting or pupation, respectively, and



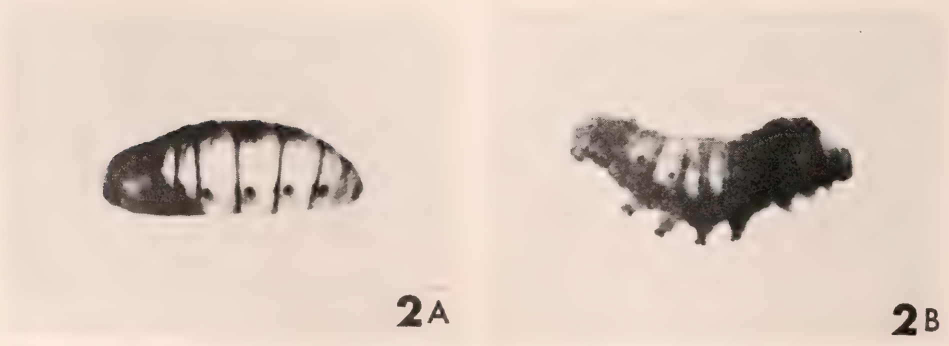


FIGURE 1. Effect of thiourea injected one day before the fourth moulting period. A, control larva (injected with KCl solution), 4 days after the injection, one day after moulting; B, larva injected with thiourea, same time after the injection; the old cuticle is retained and the swelling caused by an accumulation of fluid is visible at the dorsal side behind the head.

FIGURE 2. Effect of thiourea injected about one day before spinning. A, control (injected with KCl solution), 7 days after the injection, normal one-day-old pupa; B, insect injected with thiourea, same time after the injection; the old (larval) cuticle is retained.

TABLE I

Effect of injecting thiourea into the fourth instar larvae on the activity of phenoloxidase and the behavior and moulting of the larvae. The larvae were injected with 10 µmoles thiourea per gram body weight; the control larvae received 5 µmoles KCl/g. body weight. The activity of phenoloxidase is expressed in ml. O_2 uptake per gram wet weight (Q_{O_2}) during the first 15 minutes of measurement

Time after injection	Control larvae		Thiourea-injected larvae	
	Phenoloxidase activity	Behavior or state	Phenoloxidase activity	Behavior or state
2 hours	3.3	Feeding	1.0	Feeding
1 day	3.0	No feeding, immobile (beginning of the moulting period)	1.3	No feeding, immobile (beginning of the moulting period)
2 days	3.5	Immobile	0.9	Immobile
3 days	4.1	After ecdysis; start feeding	0.7	Swollen, the old cuticle retained, accumulation of haemolymph between the cuticles
4 days	3.6	Feeding	0.3	Swollen, the old cuticle retained, accumulation of haemolymph
5 days		Feeding		Dead

they finally developed into normal adults. However, if the injection of thiourea preceded one day or less the beginning of the last larval moulting period,[2] or the beginning of the spinning, there was an inhibition of the moulting or pupation. In the case of the fourth instar larvae, the injected insects continued to feed and then stopped feeding exactly at the same time as control larvae. The initial phase of the moulting period, *i.e.*, the "moulting dormancy," also occurred normally. However, whereas one or two days later the controls underwent the normal ecdysis, the larvae injected with thiourea developed an abnormal appearance. The old cuticle was not removed. An abundant quantity of a haemolymph-like yellow fluid accumulated between the old and the new cuticles, causing a swollen appearance of the larva, especially at its anterior part (Fig. 1). The new cuticle appeared to be abnormally soft and delicate. In a few days the larvae died (Table I), and they did not survive even if the old cuticle was cut and removed experimentally.

When the fifth instar larvae were injected with thiourea approximately one day before spinning, they formed normal cocoons and turned into normal prepupae. However, they never pupated normally. The old cuticle was not removed and a great quantity of a haemolymph-like fluid accumulated between the old (larval) and the new (pupal) cuticle (Fig. 2). Whereas the cuticle of the control pupae turned brown within a few hours following pupation, the new cuticle of the injected insects remained pale and turned yellow or light brown only on the dorsal parts of the animal. Such abnormal insects survived sometimes up to 13 days, but never developed into adults.

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[2] The term "moulting period" will be used in this paper to designate the ecdysis, or the removal of old cuticle, together with the preceding phase of immobilization ("moulting dormancy"). The last larval moulting period usually lasted two days.

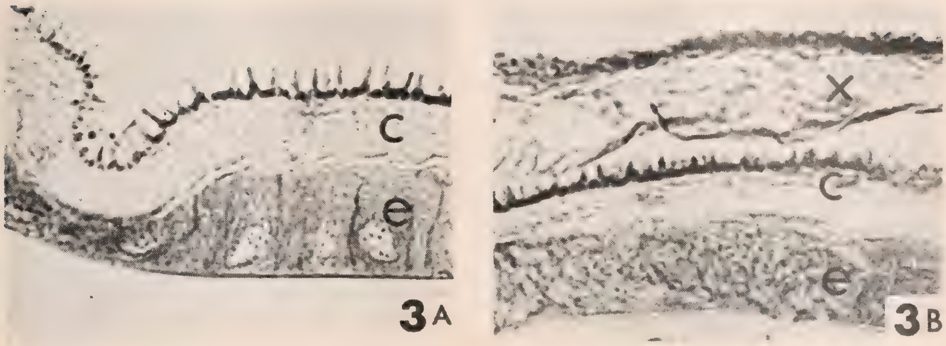
Table I shows the effect of the thiourea injection on the activity of phenoloxidase. The insects were homogenized at various times following the injection, and the activity of the enzyme was measured. In comparison with the control insects, the activity of phenoloxidase in the thiourea-injected animals was found to be strongly inhibited.

The character of these disturbances in the larval moulting and pupation caused by thiourea suggested that this compound might affect the formation of the new cuticle in such a way that it becomes more delicate and permeable to the haemolymph or, in the case of pupation, by preventing or inhibiting the sclerotization. To investigate this effect of thiourea in more detail, histological examination of the cuticle was carried out. It appeared (Fig. 3) that in the case of the fourth larval moulting no changes could be observed in the cuticle of thiourea-injected larvae as compared with the controls. The only difference was the presence of the old cuticle in the sections from thiourea-injected larvae (Fig. 3B). In the larvae injected with thiourea, as well as in the control larvae injected with KCl or not injected at all, the new cuticle developed simultaneously and appeared similar in thickness, shape and staining properties.

TABLE II

Effect of injecting thiourea into the fifth instar larvae. All details as in Table I

Time after injection	Controls		Injected with thiourea	
	Phenoloxidase activity	Behavior or state	Phenoloxidase activity	Behavior or state
1 hour	0.3	Walking	0.2	Walking
1 day	0.8	Spinning	0.1	Spinning
2 days	2.0	Cocoon partly formed	0.3	Cocoon partly formed
3 days	3.3	Cocoon completed	0.3	Cocoon completed
4 days	2.0	Prepupa	1.2	Prepupa
5 days	3.0	Prepupa	0.5	Swollen prepupa
6 days		Young (yellow or light brown) pupa		Swollen prepupa
7 days		Pupa (dark brown)		Swollen prepupa, accumulation of haemolymph between the cuticles
8 days		Pupa (dark brown)		Swollen prepupa, accumulation of haemolymph between the cuticles
9 days		Pupa (dark brown)		Swollen prepupa, accumulation of haemolymph between the cuticles
10 days		Pupa (dark brown)		Swollen prepupa, accumulation of haemolymph between the cuticles
11 days		Pupa (dark brown)		Swollen prepupa, accumulation of haemolymph between the cuticles
12 days		Pupa (dark brown)		Swollen prepupa, accumulation of haemolymph between the cuticles
13 days		Pupa (dark brown)		Dead



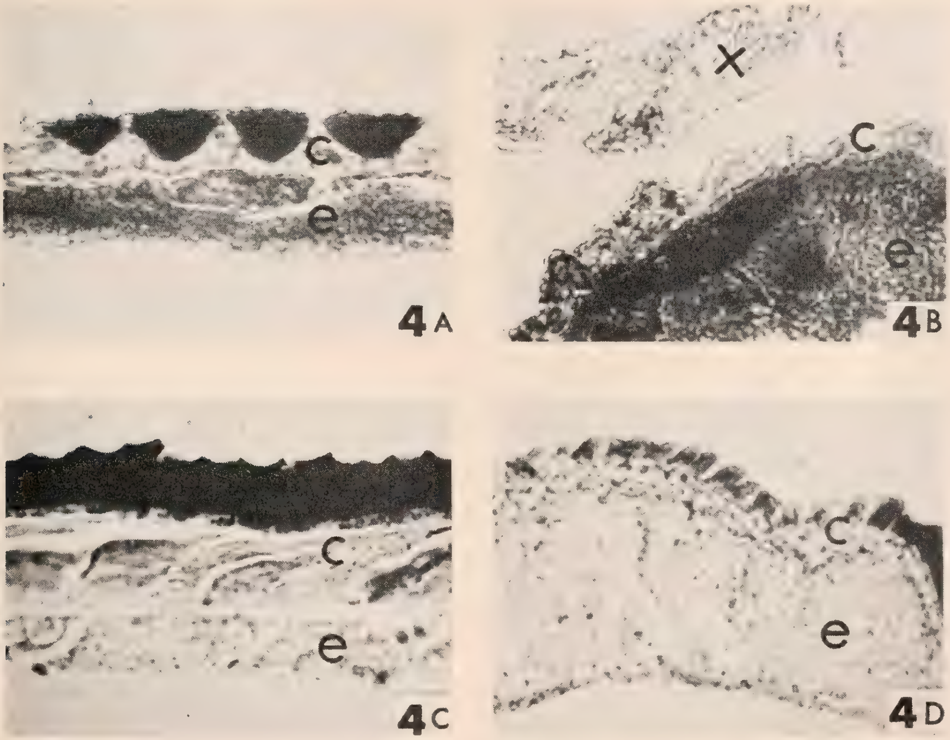


FIGURE 3. Effect of thiourea injection on the integument during the fourth larval moulting. A, control (larva injected with KCl solution), three days after the injection, shortly after moulting; B, larva injected with thiourea, same time after the injection. Indications: e, epidermal cells; c, cuticle; x, old cuticle (retained). Stained with pyronine and methyl green.

FIGURE 4. Effect of thiourea injection on the integument during pupation. A, control (injected with KCl solution), 6 days after the injection, shortly after pupation; black-coloured discs of sclerotized cuticle are clearly visible; B, insect injected with thiourea, 6 days after the injection; no sclerotization can be seen, the old cuticle is retained; C, control, 8 days after the injection of KCl solution, two days after pupation; note the thick and continuous layer of the sclerotized cuticle (black-colored); D, insect injected with thiourea, 12 days after the injection; sclerotization of the outer layer is not complete. Indications: e, epidermal cells; c, cuticle; x, old larval cuticle (retained). Stained with silver nitrate.

A quite different picture was seen in the case of the pupal moulting. Here, after injection of thiourea the inhibition of the sclerotization and pigmentation was clearly visible. In the untreated insects and in those injected with the KCl solution, the sclerotization was manifested by the formation of hard brown-colored discs in the epidermis (Fig. 4A), whereas no such discs could be seen at the same time in the insects injected with thiourea (Fig. 4B). The discs increased in size and finally joined together, forming a layer of brown cuticle staining black with $AgNO_3$ (Fig. 4C). In the insects treated with thiourea the formation of these brown discs was delayed for several days, and they were usually smaller and less numerous than in normal and control specimens. Very often, the discs did not appear at all; instead, the outer border of the epidermis turned brown, forming a thin layer of sclerotized cuticle (Fig. 4D).

DISCUSSION

The present investigation demonstrates that thiourea, when injected into silkworm larvae, disturbs the moulting process. This is manifested most conspicuously by the retention of the old cuticle. In this respect these results agree with earlier observations of Fukuda (1953) on silkworm larvae fed with mulberry leaves coated with thiourea.

Another effect of thiourea poisoning observed in the present investigation was the accumulation of haemolymph or a haemolymph-like fluid between the old and the new cuticles. Whether this was the result of tearing the new cuticle, rather than the result of filtration of haemolymph through the cuticle, cannot be decided as yet. A similar accumulation of a haemolymph-like fluid between the two cuticles was observed by Jeuniaux (1958) in larvae ligated behind the head. As is well known, ligation prevents hormones produced in the anterior part of the body (brain hormone and moulting hormone) from penetrating to the posterior part, thus preventing the moulting. It has been shown (Karlson and Schweiger, 1961) that the moulting hormone (ecdyson) increases the activity of phenoloxidase. Thus, it seems possible that the accumulation of a fluid between the two cuticles in the ligated larvae is due to some enzymatic disturbances, as in the case of the thiourea-injected larvae, rather than to the simple mechanical effect of the ligation, as suggested by Jeuniaux (1958).

As shown by the present investigation, thiourea had no visible effect on the processes of feeding and spinning, *i.e.*, during periods of a low phenoloxidase activity (Wojtczak, 1956). Abnormalities did not appear until moulting or pupation, *i.e.*, at those stages where there is a considerable increase in the activity of phenoloxidase (Wojtczak, 1956). This increase was found to be partly prevented by thiourea injected into the larval body. Thus, it seems highly probable that the abnormalities in larval moulting and pupation brought about by the injection of thiourea are mainly, if not solely, due to the inhibition of phenoloxidase.

The normal behavior of spinning larvae injected with thiourea observed in our experiments can be contrasted with the results of Jones and Wilson (1959), who found abnormalities in cocoon spinning by larvae of *Philosamia cynthia* injected with phenylthiourea. It cannot be decided whether these differences are due to a slightly different action of thiourea and its phenyl derivatives, or to a different susceptibility of *Bombyx mori* and of *Philosamia cynthia* to the poison used. It would be also interesting to investigate whether, besides abnormalities in cocoon spinning of *Philosamia*, there were disturbances in the pupation process similar to those observed in the present study.

The fact that the new cuticle of thiourea-injected silkworms was more delicate and permeable to haemolymph, and that the process of sclerotization was inhibited indicate that thiourea may cause serious morphological changes in the cuticle. Such changes, consisting of a delay and a partial inhibition of the sclerotization and pigmentation, were indeed revealed by the present investigation in the case of pupal moulting. It cannot be excluded, however, that thiourea may also induce changes and abnormalities in other organs and tissues of the insect.

In insects injected with thiourea, not only was the sclerotization of the cuticle partly inhibited, but also the cuticle itself was usually thinner than in the control insects. On the other hand, the layer of epidermal cells appeared much thicker.

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SUMMARY

1. Thiourea injected into silkworm larvae, *Bombyx mori* L., was found to inhibit the activity of phenoloxidase.

2. The effect of thiourea injection on moulting and pupation of the larvae was examined. When the injection preceded 24 hours or less the fourth moulting period or the spinning, disturbances in larval moulting and in pupation were observed, respectively. They consisted of a retention of the old cuticle and an accumulation of a haemolymph-like fluid between the old and the new cuticles. Histological examination revealed a partial inhibition and a delay of the sclerotization of pupal cuticle.

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BLOOD GROUP REACTIVE SUBSTANCES IN SOME MARINE INVERTEBRATES[1]

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This paper reports the occurrence of substances in some marine invertebrates, the specific reactions of which can be closely identified with those of known vertebrate blood grouping systems. Such substances, while found in forms as diverse as vertebrates, spermatophytes and microbes (Boyd, 1962; Springer, Williamson and Readler, 1962), have only rarely been reported in marine invertebrates, even though it is known that erythrocyte agglutinins of broad specificity can be obtained from the body fluids of various invertebrate forms (Tyler and Metz, 1945; Tyler and Scheer, 1945; Tyler, 1946; and additional references in Cushing and Campbell, 1957). In fact, in addition to the reference to "mollusca" and the "lobster" in Boyd's (1956) tabulation of the occurrence of Forsmann antigens, only the "oyster" (Springer, Rose and György, 1954) and the lobster, *Homarus americanus* (Sindermann and Mairs, 1959), appear to have been investigated from this point of view.

Invertebrates used in this present study included the sipunculid, *Dendrostomum zostericolum* (Chamberlain), the inn-keeper worm, *Urechis caupo* (Fisher and McGinitie), the spiny lobster, *Panulirus interruptus* (Randall), and *Octopus bimaculatus* (Verrill) (or *O. bimaculoides* Pickford and McConnaughey, 1949). Additional animals are referred to in the text.

Materials and Methods

Invertebrates were collected at Santa Barbara, with the exception of the inn-keeper worm which came from Newport Beach, California. Whale erythrocytes came from individuals taken off San Francisco, basking shark serum from Santa Barbara, and sea lion serum from the Coronados Islands.

Sipunculid blood samples of 2 to 4 ml. were obtained by puncturing the posterior end of worms with a 25-gauge, ¾-inch needle. Hemerythrocytes in these samples were concentrated by centrifugation. Serum and sperm and egg layers were removed by aspiration. The few sex cells and those of other types (*cf.* Triplett, Cushing and Durall, 1958) that remained among the packed hemerythrocytes were observed microscopically not to be involved in the agglutinations to be reported. Hemerythrocytes were washed according to usual serological procedures in isotonic saline (3% sodium chloride). As relatively weak reactions were obtained if the cells used had been standing for several hours, all experiments were completed within two hours after bleeding.

Reactions between sipunculid cells and serums were determined by tube agglutinations. One drop of "2%" cell suspension was mixed with three drops of serum dilution, left for 10 minutes, shaken, centrifuged for one minute, and read macroscopically according to conventional methods. Symbols used to describe reactions are as follows: ++++ symbolizes complete agglutination, and +++, ++, +, and − symbolize progressively weaker through negative reactions. Identical symbols record degrees of hemolysis where this was studied. Saline controls showed that cells never autoagglutinated. Absorptions with sipunculid cells were made at room temperature, using two volumes of serum to one volume of packed cells. Such mixtures were kept agitated for 45 minutes, centrifuged, and reabsorbed with fresh cells for 30 minutes. Guinea pig complement was used in the hemolysis of sheep cells.

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[1] This work was supported in part by a contract with the Office of Naval Research, Biology Branch (NONR 03503).

Octopuses were kept in the laboratory according to methods described by Schuyler (1961). Octopus serum was obtained by hypodermic puncture of one of the sinuses leading to the brachial hearts from animals anesthetized in MS 222. Serum samples could also be obtained from dead animals if fresh, one 60-lb. specimen being bled in this way. Cells and other suspended materials were removed by filtering or centrifuging. Agglutination tests were performed with a view to conserving serum by mixing capillary drops from tubes 1.8–1.2 × 100 mm. with a fine-point needle. Duplicate mixtures could be placed near others in a single depression on a glass plate and covered with glass sealed with Vaseline. These preparations were then put into a Petri dish with moist paper to prevent drying and shaken intermittently on a Yankee Rotator for twenty minutes. Readings were made at 10 × magnification and scored as noted above. Inhibitions were performed by mixing octopus serum and antiserum for 20 minutes, then adding cells and reading after an additional 20 minutes. Three per cent sodium chloride was used except for the washing and suspension of erythrocytes, where 1% sodium chloride was used. No effects of varying salt concentrations were observed between these two figures.

Whale erythrocytes were collected into equal volumes of glycerol-citrate during flensing and kept frozen at −20° C. until use, when they were recovered by dialysis (*cf.* Cushing, Fujino and Takahashi, 1959). Serum of the spiny lobster was obtained from the dorsal sinus, using an 18-gauge needle. Slide tests were used to observe agglutinations, which were recorded as noted above.

Reagents for these studies included anti-human blood typing lectins and serums (Hyland Laboratories, Los Angeles), the normal serums of various animals, and hetero-immune serums prepared by the injection of rabbits.

Observations were generally made at room temperatures of approximately 20° C. and the tests to be reported were repeated at least three and often several times.

OBSERVATIONS

Hemerythrocyte antigens of a sipunculid

A few invertebrates have blood cells comparable to the erythrocytes of vertebrates in that they are specialized for the carrying of respiratory pigments. The sipunculid, *Dendrostomum zostericolum*, is one of these, its cells containing hemerythrin (Prosser, 1952). Information concerning the classification, morphology and ecology of this species is given in Fisher (1952), Hyman (1959), and Peebles and Fox (1933).

Table I

Comparison of sipunculid and human cell reactions

Serum	Reciprocal of serum dilutions	Sipunculid	Human cells of types			
			A	B	AB	O
Rabbit, non-immune #4	2	–	+	–	+	–
	4	–	+	–	+	–
	8	–	+	–	–	–
Rabbit, anti-sipunculid #4	2	++++	++++	+	+++	–
	4	+++	+++	+	++	–
	8	+	++	–	+	–
	16	–	+	–	+	–
	32	–	+	–	+	–
Basking shark #60	0	++++	++	–	+++	–
	2	++++	++++	–	++++	–
	4	+++	++++	–	++++	–
	8	++	++++	–	++++	–
	16	–	++++	–	++++	–
	32	–	++++	–	+++	–
Sea lion #3	0	++++	++++	++	+++	++
	2	++++	++++	++	++++	+
	4	++	++++	++	++++	+
	8	+	++++	+	++++	+
	16	–	+++	–	++++	–
	32	–	++	–	+++	–
Anti-A	0	++++	++++	–	++++	–
	2	+++	++++	–	++++	–
	4	++	++++	–	++++	–
	8	+	++++	–	++++	–
	16	–	++++	–	+++	–
	32	–	++++	–	+++	–
Anti-B	0	+++	–	++++	++++	–
	2	++	–	++++	++++	–
	4	+	–	++++	++++	–
	8	+	–	++++	++++	–
	16	–	–	++++	++++	–
	32	–	–	++++	++++	–
Anti-white croaker #1	0	+++	–	–	–	–
	2	+++	–	–	–	–
	4	++	–	–	–	–
	8	+		–	–	–

Reactions of sipunculid hemerythrocytes, and human erythrocytes of various types with different kinds of unabsorbed serums. Explanation of method of testing and scoring will be found in the text.

Preliminary observations were made on the reactions of several serums with washed sipunculid cells. Of these, sheep, cow, and horse serums gave positive reactions, while lobster serum gave negative ones. Rabbit normal serums were very weakly positive, or negative, as was human Rh typing serum (anti-Rh$'_0$ (CD)), and rabbit anti-human M and N. The reactions of additional serums are given in Table I. These show that rabbits can produce antibodies reactive with sipunculid cells, and that these serums also react with human cells that carry the A specificity. Reactions with normal basking shark (*Celorhinus maximus* Gunner) and California sea lion serums (*Zalophus californianus* Dall) point to a similar correlation, as do those with human anti-A. The reactions of anti-B typing serums suggest that additional antigens occur, and rabbit anti-white croaker (*Genyonemus lineatus* Ayres) serum shows that sipunculid cells have at least one unique specificity within the framework of our observations.

Table II gives examples of the results of absorptions of basking shark serum with various cells which confirm a specific relation between the affinities of sipunculid and human type A cells for a fraction of the shark antibodies. Additional data showed that the antibodies involved did not distinguish between human cells phenotyped as A_1 or A_2.

Table III gives examples of the results of absorbing human typing serums. Absorptions of anti-A showed that human cells carrying A, B and O antigens removed almost all antibodies reacting with sipunculid cells (even when A absorptions were incomplete) while sipunculid cells did not remove any antibodies reacting with A cells.

Absorptions of anti-B serum with these same cells had little or no effect on the antibodies reactive with sipunculid cells, nor did sipunculid cells remove any anti-

TABLE II

Sipunculid and human A cell reactions with absorbed basking shark serum

Cells tested	Reciprocal of serum dilutions	Serum absorbed by cells of				
		None	Sipunculid	Human types		
				A	B	O
Sipunculid	0	++++	–	+++	++++	++++
	2	+++	–	++	+++	+++
	4	++	–	+	+++	+++
	8	++	–	–	++	++
	16	+	–	–	+	+
	32	–	–	–	–	–
Human type A	16	++++	++++	–	++++	++++
	32	+++	+++	–	++++	++++
	64	+++	+++	–	+++	++++
	128	++	+	–	+++	+++
	256	+	–	–	++	++
	512	+	–	–	+	+

The agglutination of various kinds of cells by unabsorbed and absorbed basking shark serum. Explanation of methods of testing and scoring will be found in the text.

Table III

Sipunculid and human cell reactions with absorbed anti-A and anti-B sera

Cells tested	Reciprocal of serum dilutions	Serum absorbed by cells of				
		None	Sipunculid	Human types		
				A	B	O
Anti-A						
Sipunculid	0	++++	−	+	+	+
	2	+++	−	−	−	+
	4	++	−	−	−	−
	8	+	−	−	−	−
	16	−	−	−	−	−
Type A	16	++++	++++	+	++++	++++
	33	++++	+++	++	+++	+++
	64	++++	+++	++	+++	+++
	128	+++	+++	−	+++	+++
	256	++	++	−	++	++
	512	+	+	−	+	+
	1024	+	+	−	−	−
Anti-B						
Sipunculid	0	++++	−	++++	++++	++++
	2	++++	−	+++	++++	+++
	4	+++	−	+	+++	+
	8	+	−	−	+	−
	16	−	−	−	−	−
Type B	2	++++	++++	++++	−	++++
	4	+++	++++	++++	−	++++
	8	+++	++++	+++	−	++++
	16	++	+++	++	−	+++
	32	++	+++	++	−	++
	64	+	++	+	−	+
	128	−	+	+	−	+

The agglutination of various kinds of cells by unabsorbed and absorbed human blood typing serums. Explanations of methods of testing and scoring will be found in the text.

bodies reacting with B cells. Two serums from type AB humans failed to agglutinate sipunculid cells while a third did so very weakly. No observations were made on serums from type O persons.

That human cells removed antibodies which agglutinated sipunculid cells without themselves being agglutinated by these antibodies is not unexpected in view of related kinds of experiences by other workers (*e.g.*, Stormont and Suzuki, 1960; Cushing, Fujino and Calaprice, 1963; Stone, 1962; Pirofsky, Cordova and Imel, 1962). Further analysis of the reactions just reported should of course include considerations of the effects of temperature, the specific relation between serum and cell sources, the genotypes of persons providing these materials, and the multitude

TABLE IV

Forssman reactions of sipunculid cells and anti-sipunculid serums

(a) Hemolysis of sheep erythrocytes by anti-sipunculid hemerythrocyte antiserum

Rabbit #5, non-immune control serum		Rabbit #5, anti-sip. antiserum	
Dil.	Hemolysis	Reciprocal of serum dilutions	Hemolysis
0	–	0	++++
2	–	2	++++
4	–	4	++++
8	–	8	++
16	–	16	+
32	–	32	–

(b) Hemolysis of sheep erythrocytes by anti-Forssman antiserum

Anti-Forssman antiserum #1 not absorbed		Anti-Forssman antiserum #1 absorbed with sip. cells	
Dil.	Hemolysis	Dil.	Hemolysis
16	++++	16	++++
32	++++	32	+++
64	++++	64	+++
128	++++	128	++
256	++	256	–
512	+	512	–

(c) Agglutination of sipunculid cells by anti-Forssman antiserum

Non-immune control serum #1		Anti-Forssman antiserum #1	
Dil.	Agglutination	Dil.	Agglutination
0	–	0	++++
2	–	2	++++
4	–	4	++++
8	–	8	+++
16	–	16	+
32	–	32	–

Various tests showing that sipunculid cells have Forssman antigen. Explanation of methods of testing and scoring will be found in the text.

of complexities that have been shown to exist among agglutinogens and the heterogeneous antibodies used to identify them (*cf.* Owen, 1954, for a discussion of this subject).

Table IV shows that sipunculid cells conform with the criteria accepted by Stormont and Suzuki (1958) for establishing the presence of Forssman antigen. Their criterion is that cells carrying this antigen should cross-react with and be able to absorb out some (not necessarily all) of the sheep cell hemolysins present in an antiserum prepared in rabbits by the injection of guinea pig organs. Such cells should also cause the production of sheep cell hemolysins in rabbits.

In conformance with observations of Stormont and Suzuki, human A cells did not absorb antibodies from our Forssman antiserum. Sipunculid cells, however, did remove some of these antibodies, providing further evidence for the occurrence of Forssman specificity in this species. (Note that lysis of sipunculid cells did not occur with either Forssman or homologous antiserums.)

Hemocyte reactions in a second invertebrate: Urechis caupo

The inn-keeper worm, *Urechis caupo* (Fisher and McGinitie), is a burrowing worm of the phylum Echiuroidea. It is another of the few invertebrate animals whose blood contains pigmented blood cells. In this case, the respiratory pigment is a hemoglobin.

One specimen of this species was obtained through the courtesy of Dr. Albert Tyler, of the California Institute of Technology. Its cells were weakly agglutinated by normal serum from horse, pig, sheep, cow, and turkey. They were not agglutinated by normal serums from the sea lion, porpoise, basking shark, sipunculid, and rabbit. In addition, they did not react with anti-A and anti-B blood typing reagents or with rabbit anti-sipunculid hemerythrocyte antiserum. Therefore, it is apparent that *Urechis* cells differ markedly in their antigenic properties from those of *Dendrostomum.*

Agglutinins

The classical work on invertebrate agglutinins is that of Tyler and his associates in their studies on the serum of the spiny lobster (Tyler and Metz, 1945; Tyler and Scheer, 1945). Among their observations was the finding that several agglutinins were present in this serum, each capable of reaction with antigens on erythrocytes or sperms that were Class-specific among the wide range of organisms tested.

More recently, Sindermann and Mairs (1959) have shown that the unabsorbed serum of the eastern lobster (*Homarus americanus*) differentiates between the erythrocytes of individual Atlantic sea herring (*Clupea harengus harengus* Linnaeus), agglutinating the cells of some fish to titers of 1 in 128, and others only to 1 in 4. This serum has been utilized as a blood typing reagent in studies on herring.

We have found that serum from the spiny lobster is capable of differentiating individual whales within at least four different species. The blood group system involved appears to be the Ju system originally described by Fujino (1953) in finback whales and now known to be widely distributed through the Cetacea. This system consists of a major pair of antigens that determine the three phenotypes Ju1, Ju2 and Ju1·2. The two antigens are detected by properly absorbed rabbit heteroimmune serums. In addition, the Ju2 antigen reacts with "natural" agglutinins in the serums of whales and several species of domestic animals, exists as a series of subtypes, and, at least in the sperm whale, appears to have solubility properties not unlike those typified by the J substance of cattle. Recent papers introducing the literature on these antigens will be found in Fujino (1962), and Cushing, Fujino and Calaprice (1963).

TABLE V

Agglutination of whale erythrocytes by horse and spiny lobster serums

Species	Cells	Horse serum dilutions					Lobster serum dilutions				
		2	10	20	40	80	Und.	2	4	8	16
Sperm	25	++++	++++	++++	+++	++	+	+++	+++	++	−
	* 30	−	−	−	−	−	−	−	−	−	−
Humpback	65	++++	++++	++++	++++	+++	+++	++++		+++	+
	194	++++	++++	++++	++++	+++	++	++++		−	−
	36	++++	++++	++++	++++	+++	+	++		+	++
	153	++++	++++	+++	+	+	−	−		−	−
	132	+++	++	+	+	−	−	−		−	−
	*111	−	−	−	−	−	−	−		−	−
Sei	123	++++	+++	+++	++	+	+	++	++	+	−
	126	++++	++++	+++	++	+	−	+	++	−	−
	122	++++	++++	+++	++	+	−	−	−	−	−
	*136	−	−	−	−	−	−	−	−	−	−
Finback	198	++++					++++	++++	+++	+++	++
	26	++++					+	++	+++	++	+
	23	++++					+	+	+	+	+
	* 37	−					−	−	−	−	−
Blue	*	−					−	−	−	−	−

Examples showing the reactions of whale erythrocytes with horse and lobster serums. Cells reacting with horse serum have the Ju2 antigen, and reacted to different degrees with lobster serum. Cells that did not react with horse serum lack the Ju2 antigen and failed to react with lobster serum (cells marked with an asterisk are examples of all such negative cells). Total number of observations from which examples were taken in this series as follows: sperm whales, one Ju2 positive, nine negative. Humpback whales, five Ju2 positive, thirty-two negative. Sei whales, three Ju2 positive, five negative. Finback whales, eight Ju2 positive and twenty-three negative. Blue whales, one negative.

Table V gives examples of the reactions of whale erythrocytes with spiny lobster serum. These show that this serum has potential value as a sub-typing reagent for the Ju2 antigen, and give support to the prediction of Tyler (1961, p. 491) that lobster serum will continue to yield substances of immunological significance.

Inhibition of human anti-A serum by Octopus serum

Studies made in conjunction with transplantation experiments on *Octopus bimaculatus* (Trump and Cushing, reported by Cushing, 1963) showed that serum of this species did not agglutinate various human erythrocytes known to be carrying the A, B, O, M, and N antigens. Octopus serum was therefore tested for its ability to inhibit commercial typing serums (Hyland Laboratories) reactive with these antigens. No inhibition was observed with respect to anti-B, anti-M, anti-N, anti-H (against O and A_2 cells), or Anti-A_1 lectin (*Dolichos bifloris*). However, isoimmune anti-A was partially inhibited with respect to its reactions with type A human cells. Table VI shows examples of many protocols that consistently demonstrated this inhibition to involve both A_1 and A_2 cells. Relatively more inhibition

Table VI

Specific inhibition of human anti-A blood typing serum by octopus serum

	Reciprocal of serum dilutions						
	Not diluted	2	4	8	16	32	Saline control
Human type A_2 cells (individual one)							
Saline added	+++	++++	+++	++	+	+	–
Octopus serum added	+++	+++	+	–	–	–	–
Human type A_2 cells (individual two)							
Saline added	++++	++++	++++	++	+	+	–
Octopus serum added	+++	++	+	–	–	–	–
Human type A_1 cells (individual three)							
Saline added	+++	+++	++	++	++	++	–
Octopus serum added	+++	+++	++	++	+	–	–
Human type A_1 cells (individual four)							
Saline added	++++	++++	++++	+++	++	++	–
Octopus serum added	++++	++++	+++	+++	+	+	–

The above protocols are selected examples of many experiments that consistently showed that octopus serum partially inhibits the agglutination of human type A cells by human anti-A serum, and that relatively more inhibition occurred with A_2 cells than with A_1 cells.

for the agglutination of A_2 cells was consistently indicated, but quantitative confirmation of this point has yet to be made. The inhibitory power of octopus serum disappeared when the serum was diluted to 1 in 32, but was not destroyed when the serum was heated sufficiently to cause protein coagulation (70° C. for 12 minutes). Efforts to absorb and to conjugate the soluble substances onto guinea pig and type O human cells gave some indications of success, but were not carried to a conclusive point.

Other marine invertebrates have not been previously investigated for soluble inhibitors of blood typing reagents with the exception (references in the introduction) of an unidentified species of "oyster." Water extracts of this form contained a non-dialyzable substance capable of inhibiting isoantibodies for human A, and eel anti-H while not inhibiting anti-B. Some cursory examinations of invertebrate serums in our laboratory either yielded negative results or were complicated by the presence of agglutinins and could not be pursued within the time available.

Discussion

The observations reported above are significant not so much individually as collectively in that they support the concept of the ubiquitousness among organisms of substances with specificities and reactivities closely akin to those initially and classically described for the A, B, and O blood grouping antigens and isoantibodies. This concept continues to be involved with such intriguing questions as that of the physiological significance of these widely distributed substances, and that of the biochemical basis for the evolution of the antibody response. Additionally, a better understanding of these substances may lead to their use as genetic markers in invertebrate populations, such, for example, as among the plankton. Markers of this sort are already of established value in distinguishing genetically isolated populations within single species of marine animals, and have great potential value as aids in learning something of the ecological and evolutionary forces that influence these populations (*cf.* a "Symposium on immunogenetic concepts in marine population research" 1962, and Manwell, 1963).

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Summary

The occurrence of blood group reactive substances in some species of marine invertebrates is reported. These include:

1. Antigens on the hemerythrocytes of the sipunculid, *Dendrostomum zostericolum,* that react with various antiserums including human anti-A, human anti-B and anti-Forssman, and a comparison of these reactions with those of the hemocytes of the inn-keeper worm, *Urechis caupo.*

2. A substance in the serum of the spiny lobster, *Panulirus interruptus,* that agglutinates erythrocytes carrying the Ju2 antigen in whale species. These include the sperm, humpback, sei and finback whales.

3. A substance in *Octopus bimaculatus* serum that specifically inhibits the agglutination by anti-A serum of human cells carrying the A_1 and A_2 antigens.

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A COMPARISON OF THE PHYSIOLOGY AND ECOLOGY OF THE ESTUARINE ISOPOD CYATHURA POLITA IN MASSACHUSETTS AND GEORGIA[1]

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The rate of raciation and speciation in estuarine species may differ from that of organisms from other environments. Estuaries are characterized by great fluctuations of environmental factors, but since these fluctuations are a permanent feature of the environment, it can be argued that "the aggregate rate of change in the estuarine environment may actually be less than in the geographical province in which it occurs over the same period of time" (Hedgpeth, 1957, p. 695). Simpson (1944) recognized that an organism which adapted to cyclic environmental variations might continue unchanged during climatic changes. This concept indicates that estuarine species may be evolutionarily conservative. Some estuarine forms, such as oysters, gastropod stocks of the Neritacea, and the horseshoe crab, *Limulus,* have persisted for long periods of time relatively unchanged, and may be examples of this evolutionary conservatism (Hedgpeth, 1957).

Species of the isopod genus *Cyathura* are found in estuaries all over the world (Burbanck, 1959). Miller and Burbanck (1961) have studied a species which is widely distributed along the Atlantic and Gulf Coast seaboard of the United States and have established for it the new combination *Cyathura polita.* They noted intraspecific variations in the apex of the appendix masculinum and in the degree of fusion of the anterior 5 pleonites but did not attempt to correlate these variations with the location of the population which showed them. Burbanck and Burbanck (1961) have shown that a geographically correlated intraspecific variation occurs in the dorsal chromatophore pattern. They found one type of pattern in animals collected north of the Hudson River, and another in animals collected from New Jersey southward. On the basis of these results they have suggested (p. 263) ". . . that there might be populations which could be considered distinct races within the species."

The present study was undertaken to evaluate further the variability of the species by comparing two widely separated Atlantic coast populations. A population located in an estuarine meander in Marshfield, Massachusetts, was compared with one in a sandy beach on Sapelo Island, Georgia. Measurements were made of osmoregulatory behavior and oxygen consumption of animals from each population at different temperatures and over a wide range of salinities, and data were collected

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on such ecological features as the habitat niche, population parameters, and the fauna associated with the cyathurans.

MATERIALS AND METHODS

Both populations compared in this study were observed at two times of the year. The Sapelo population was studied throughout the fall of 1961, and was revisited in March, 1962. The Marshfield population was studied in the summer of 1961, throughout the spring of 1962, and again in July and August, 1962. Ecological observations were made whenever the two habitats were visited. Physiological experiments were run on the Sapelo population in the late fall of 1961 and in March, 1962, and on the Marshfield population in the early spring of 1962 and again in July.

Ecological Methods

Habitat observations

The habitat of each population was observed and photographed, and salinity and temperature measurements were made. The salinity was measured by silver nitrate titration. At Sapelo the Mohr method (Barnes, 1959) was used, while at Marshfield, a simplified technique (Barnes, 1959, p. 95) was substituted. The temperature was measured with a Rochester bimetal helix dial thermometer.

Salinity measurements were made in each area at high and low tide. At Sapelo the salinity of the water overlying the beach at high tide and of the seepage water in the beach at low tide was measured. These measurements were made several times in the fall of 1961, and were repeated on March 29, 1962. At Marshfield, the high- and low-tide salinity was measured at the extremes of the cyathuran's range on a spring tide June 30, 1962, and on the following neap tide, July 11, 1962. The temperature of the water in which the cyathurans were living was measured several times during the study of each area.

Population sampling

The dispersion pattern and density of the cyathuran populations were measured by the use of quantitative collecting techniques. At Sapelo Island, sand from 0.1-m^2. quadrats was sieved through screening of 1.5-mm^2. mesh, the large animals retained in the screen, and the cyathurans picked out and counted. At Marshfield samples were collected from the bottom of the estuary with a Hayward Dwarf orange peel #1 grab (Burbanck, Pierce and Whiteley, 1956) and the cyathurans sieved out in the same manner. The area of the grab samples varied somewhat but averaged 425 cm^2. The counts of the cyathurans from each quadrat or grab sample were used to calculate the density and the dispersion pattern of the populations.

The length and sex of each cyathuran collected were determined. The length was measured from the base of the antennae to the tip of the telson. The sex was determined by microscopic examination of the antennae and of the second pleopod, which, in males, carries an appendix masculinum (Miller and Burbanck, 1961). These data were used to calculate the mean size of the animals and the sex ratios in the populations. Since the sex of juveniles cannot be positively determined externally, they were not included in sex ratio calculations.

Faunal association sampling

Animals were collected from each habitat, using the quantitative techniques described above. At Sapelo Island fifty 0.1-m^2. quadrats were collected within the cyathuran population, while at Marshfield 10 grab samples were taken at each of 10 stations within the range of population.

The animals found in each quadrat or grab sample were identified and counted, and the numerical ranking, density and frequency of each species calculated. The density was determined as the mean number of animals collected per 0.1-m^2. The frequency was calculated differently in the two areas. At Sapelo it was calculated simply as the percentage of quadrats in which the species was found. At Marshfield, however, most of the species occurred only in a limited part of the range of the cyathurans; therefore their frequency was determined as the percentage of samples in which they occurred compared to the number of samples taken in their part of the range. Thus if a species was collected at only three stations, its frequency was calculated on the basis of 30 samples.

A number of each species were brought back to the laboratory for positive identification and weight analysis. The identifications were validated by taxonomic experts. The ash-free dry weight of each species was obtained by drying several animals at 105° C. for 24 hours, weighing them, then igniting them at 475° C. for 20 minutes, and reweighing. The difference between the weight after drying and the weight after igniting was used as a measure of the ash-free dry weight of the samples. An average specimen weight was calculated and used to obtain an estimate of the ash-free dry weight of the species. Numerical and weight percentages were calculated for each species in the associations and cumulative percentages determined.

Physiological Methods

The cyathurans used in the physiological experiments were collected daily. At Sapelo Island they were collected at mid-tide from an open area of beach near the middle of the population. At Marshfield they were collected at low tide from the bottom of the South River, near a railroad embankment about 300 meters below the upstream limit of the population. About 1000 animals were used in the experiments.

Osmoregulatory behavior

The technique used to determine osmoregulatory behavior of *Cyathura polita* was a modification of a method described by Jones (1941) and Gross (1954). This method measures the concentration of body fluid solutes by determination of freezing point depression.

Cyathurans were acclimated for 24 hours to varying salinity and temperature conditions in fingerbowls which contained 100 ml. of water and a number of 4-mm. glass boiling beads. The fingerbowls were kept in constant temperature water baths throughout the acclimation period. The temperature of these baths was set at 15 and 30° C.; however, the temperature of the water in the fingerbowls was also affected by the room temperature. The experimental temperatures at Sapelo Island were 17 ± 0.5° C., and 27 ± 0.7° C.; at Marshfield corresponding temperatures were 15 ± 0.5° C., and 27 ± 0.7° C. The water to which the animals were acclimated was collected near the populations, and its salinity was adjusted either by diluting with distilled water or by concentrating with gentle heat. In experiments run at Sapelo Island at 17° C., salinities were 1.02, 4.0, 8.67, 12.66, 17.1, 20.7, 24.7, 26.2, 28.18, 31.1, and 34.82‰. In experiments run at Marshfield at 15° C. salinities were 1.2, 4.4, 11.42, 17.8, 24.2, 27.6, 29.4, 33.8, and 43.7‰. In experiments run at 27° C., these salinities were 1.2, 4.65, 11.0, 17.75, 22.0, 27.0, 30.2, and 35.5‰.

Body fluid samples were withdrawn from six randomly selected animals in each acclimated group, and the freezing point depression of the samples determined by comparison with solutions of known freezing point. The raw data on the body fluid freezing point depressions were analyzed to provide a mean and standard error value for each experimental group. These values were then plotted against the salinity to which the animals had been acclimated to provide a graphic representation of the osmoregulatory behavior.

Oxygen consumption

The technique used to measure the oxygen consumption of *Cyathura polita* was a modification of the water bottle method described by Riley and Gorgy (1948), and by Conover (1956). This technique measures oxygen consumption by determination of the amount of oxygen removed from a bottle by an experimental animal in a measured time. It was chosen for use in this study primarily because it does not require that the experimental vessels be agitated during the course of the experiment.

The oxygen consumption experiments were run at temperatures of 15° C. and 30° C., and at a number of salinities. At Sapelo Island these salinities were 1.02, 4.54, 8.63, 12.48, 17.1, 20.6, 24.21, 29.45, and 35.94‰. At Marshfield they were 1.0, 3.85, 10.4, 15.9, 21.7, 24.2, 27.6, 30.1, and 36.9‰. The animals were acclimated to the experimental conditions of temperature and salinity for 24 hours in fingerbowls similar to those described above.

The experimental vessels were 125-ml. glass-stoppered bottles. In each experiment 8 bottles were flushed with water of the experimental salinity, and randomly selected, acclimated animals of uniform size were introduced into 6 of them. Two bottles were used as controls. All eight bottles were stoppered and placed in a constant temperature bath set at the temperature to which the animals had been acclimated.

The bottles were left in the bath for 24 hours before they were removed and the oxygen concentration of each determined by Winkler titration (Barnes, 1959). The difference between the oxygen concentration in the control bottles and in each experimental bottle was taken as the decrease caused by the respiration of the experimental animal. This concentration difference was used to calculate the volume of oxygen consumed per animal. The ash-free dry weight of the experimental animals was determined by the method described above and used to calculate the oxygen consumption rate in terms of milliliters of oxygen consumed per gram of ash-free dry weight per 24 hours. These data were statistically analyzed to obtain mean and standard error values and plotted against salinity.

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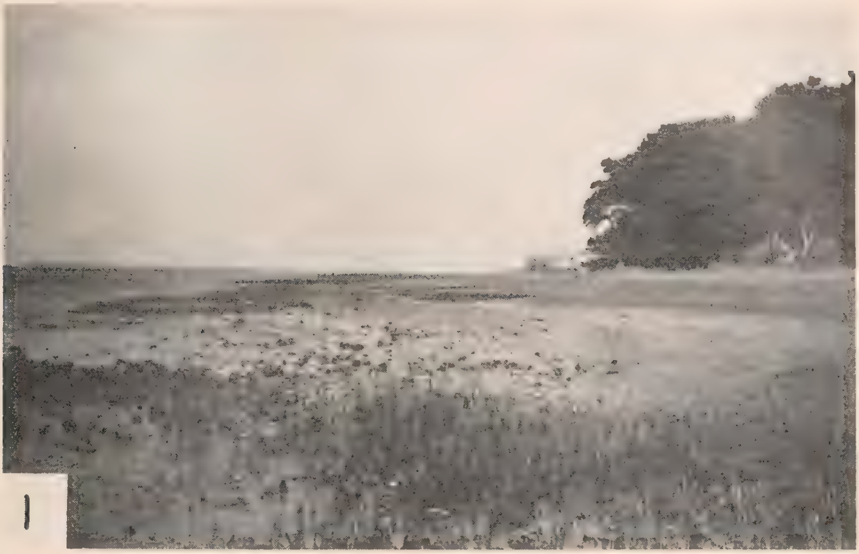

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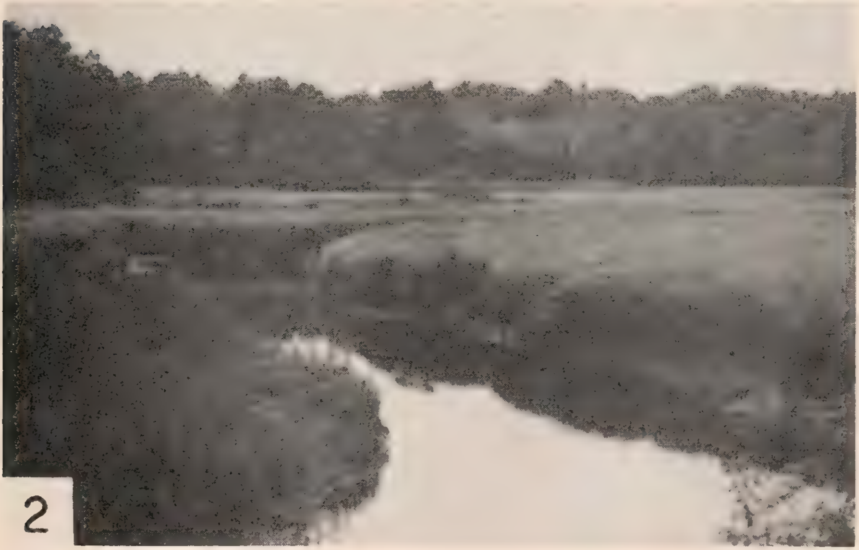

FIGURE 1. A photograph of the beach area on Sapelo Island, Georgia, which was inhabited by the southern population of *Cyathura polita*. The photograph was taken at low tide from a point near the lower edge of the intertidal zone. The cyathurans were found living among the sediment particles on the sandy beach.

FIGURE 2. A photograph of the South River, the estuary in Marshfield, Massachusetts, which was inhabited by the northern population of *Cyathura polita*. The photograph was taken at low tide from a point near the upstream limit of the population looking downstream. The cyathurans were found living among the sediment particles on the river bottom.

RESULTS

Ecological Results

The habitats

The habitat of the southern population was a sandy beach located between a steep bluff and a mudflat on the northwestern shore of Sapelo Island, Georgia (Fig. 1). The cyathurans were found in the surface layer of the beach to a depth of 5 centimeters. This layer was a mixture of sand and shell fragments and was underlain by rather impervious muddy-clay. The clay caused water to be retained in the beach at low tide and even on the hottest days the exposed surface remained damp. Many areas of the beach supported stands of the salt marsh grass, *Spartina alterniflora*.

The water which flooded the beach was turbid, well aerated and had wide fluctuations of salinity and temperature. The salinity of the water usually remained relatively constant throughout the tidal cycle, but fluctuated seasonally and during rain storms. During the fall of 1961 the salinity varied between 28.4‰ at high tide and 29.0‰ at low tide. During rainy weather the salinity of the beach water was reduced and during a shower on November 14, 1961, it fell to 25.8‰. The winter and spring rains cause an annual salinity fluctuation and on March 29, 1962, the high and low tide salinities of the beach water were 21.2‰ and 21.9‰, respectively. The temperature range of the water was extensive. During the fall of 1961 this range included a high of 30° C. and a low of 12° C. The turbidity of the water was extremely high throughout the period of study. Usually one could not see more than 15 centimeters into it.

The habitat of the northern population was the South River, a small estuarine meander in Marshfield, Massachusetts. This river becomes recognizable as it leaves the Marshfield World War II Memorial. From this point it runs about 0.5 mile through a fresh-water swamp (Shaler, 1885) dominated by *Typha* species, and about 7.75 miles through a typical salt marsh (Miller and Egler, 1950) dominated by *Spartina alterniflora* and *S. patens*, to a mouth which it shares with the larger North River. The cyathurans were found in the sediments on the river bottom. Their range began about 4.5 miles from the mouth and continued upstream for about 3.5 miles. The bottom of the river was sandy, although larger particles occurred with the sand upstream and smaller particles downstream. Figure 2 is a photograph of a section of the South River.

The water in the South River habitat was characterized by wide daily fluctuations in salinity and wide yearly fluctuations in temperature. The salinity varied from a low of 0.1‰ (low tide at the top of the range) to a high of 28.9‰ (high tide at the bottom of the range). The salinity extremes at the top of the range on a spring tide June 30, 1962, were 2.2‰ at high tide and 0.2‰ at low tide; on a neap tide July 11, 1962, these values were 0.3‰ and 0.1‰, respectively. At the bottom of the range the salinity extremes on the spring tide were 28.9‰ and 9.4‰ and 24.5‰ and 6.6‰ on the neap tide. The yearly temperature range in the habitat was extensive. A low temperature of 8° C. was measured in April, 1962, and a high of 26° C. in August, 1962.

The populations

The individuals in both populations were distributed in irregular, non-random patterns with a high degree of clumping. This clumping was recognized when the collection data were statistically analyzed, and when animals were being collected for physiological experimentation. Large groups of cyathurans were collected from some areas, while superficially similar areas yielded very few.

The sex ratio of both populations was dependent on the time of year. When 47 animals from the Sapelo population were examined on March 30, 1962, 24 were males and 23 were females; when 105 animals from the Marshfield population were examined on May 10, 1962, 49 were males and 56 were females. However, no animals with male characteristics were found when 80 animals from the Sapelo population were examined on November 3, 1961, and only 4 animals with male characteristics were found when 55 animals from the Marshfield population were examined on August 27, 1962. Intermediate periods occurred at intermediate seasons in which there were more animals with female characteristics than with male. A ratio of 32 females to 18 males was found in the Sapelo population on December 11, 1961, and a ratio of 39 females to 11 males in the Marshfield population on July 11, 1962.

Cyathurans collected at Marshfield May 10–16, 1962, were significantly larger than those collected at Sapelo Island on October 6–28, 1961 (mean length 13.3 ± 0.3 mm. as compared with 9.3 ± 0.6 mm.). Male animals collected at Marshfield were significantly larger than females. The lengths of the two sexes were 15.6 ± 0.2 mm. and 12.1 ± 0.3 mm., respectively.

The faunal associations

The data on the animals which were found in the two habitats are presented in Tables I and II. Both associations were made up of a small number of species.

TABLE I

Faunal association, Sapelo Island

Species	No. Coll.	% No. Coll.	Cumulative %	Density No./ 0.1 M.	Frequency	Ash-Free Dry Wt.	% Dry Wt.	Cumul. Dry Wt. %
Nassa (= *Nassarius*) *obsoleta*	520	52.6	52.6	10.4	84%	9.75	53.1	53.1
Cyathura polita	158	16.0	68.6	3.16	88%	0.23	1.3	54.4
Uca pugilator	109	11.0	79.6	2.18	52%	3.64	19.8	74.2
Gammarus sp.	50	5.1	84.7	1.0	50%	0.06	0.3	74.5
Laeonereis culveri	43	4.3	89.0	0.87	48%	0.57	3.1	77.6
Oligochaeta	32	3.2	92.2	0.64	44%	0.42	2.3	79.9
Nereis succinea	22	2.2	94.4	0.52	36%	0.29	1.5	81.4
Littorina irrorata	19	1.9	96.3	0.40	28%	0.30	1.5	82.9
Scoloplos fragilis	17	1.7	98.0	0.34	18%	0.23	1.2	84.2
Mercenaria mercenaria	7	0.7	98.7	0.14	14%	2.89	15.7	99.8
Haustorius sp.	6	0.6	99.3	0.12	12%	0.07	0.2	100.0
Dipteran larvae	4	0.4	99.7	0.08	8%			
Pagurus longicarpus	2	0.2	99.9	0.04	4%			

TABLE II

Faunal association, Marshfield

Species	No. Coll.	% No. Coll.	Cumulative %	Density No. 0.1 M.	Frequency	Ash-Free Dry Wt.	% Dry Wt.	Cumul. Dry Wt. %
Scolecolepides viridis	860	66.8	66.8	25.3	85%	4.3	33.0	33
Cyathura polita	290	22.4	89.2	6.82	76%	0.6	4.6	37.6
Gammarus tigrinus	100	7.8	97.0	2.4	23%	0.08	0.4	38.0
Nereis virens	11	0.9	97.9	6.47	23%	0.09	0.6	38.6
Anguilla rostrada (elver)	9	0.7	98.6	6.9	13%	1.82	14.0	52.6
Scoloplos fragilis	6	0.5	99.1	6.3	20%	0.04	0.3	52.9
Glossiphonia complanata	5	0.4	99.5	10.6	30%	0.05	0.3	53.2
Anodonta implicata	3	0.2	99.7	6.4	30%	6.13	47.0	100.0
Dipteran larvae	2	0.1	99.8	4.3	20%			
Crago septemspinosus	1	0.1	99.9					
Mya arenaria	1	0.1	100.0					

Some of these, *Cyathura polita* and *Scoloplos fragilis*, were found in both associations. Others, such as *Gammarus tigrinus* at Marshfield and *Gammarus* sp. at Sapelo, *Nereis virens* at Marshfield and *Nereis succinea* at Sapelo, were closely related to each other. Unidentified dipteran larvae were found in both areas.

Tables I and II make it clear that each association was made up of a few common species and several relatively rare species. Thus in the Sapelo Island association *Nassa* (= *Nassarius*) *obsoleta*, *Cyathura polita*, and *Uca pugilator* made up 79.6% of the animals collected, and in the Marshfield association *Scolecolepides viridis*, *Cyathura polita* and *Gammarus tigrinus* made up 97%.

Physiological Results

Osmoregulatory behavior

The results of the experiments on the osmoregulatory behavior of *Cyathura polita* are summarized in Figure 3. The cyathurans from both populations maintained their body fluids hypertonic to their environment at salinities between 1 and about 28‰. However, at salinities between 28 and 43‰ the body fluids were isosmotic with the environmental water.

The level at which *Cyathura polita* maintained its body fluid concentration changed seasonally. In December, the Sapelo animals maintained their body fluid concentration constant at a freezing point depression (Δ_f) of about −1.8° C. In March, this concentration level decreased to a Δ_f between −1.6° C. and −1.7° C. In April, Marshfield animals maintained a constant body fluid concentration at a level between Δ_f of −1.4 and −1.6° C. In July this level rose to a Δ_f over −1.6° C.

Oxygen consumption

The results of the experiments on oxygen consumption are summarized in Figure 4. The cyathurans had a relatively constant oxygen consumption rate over a salinity range from 1 to 37‰. The results of each experiment were compared statistically to the results of the experiment run at the next higher and lower salinity and, in 29 of the 32 cases compared, no significant difference was found.

No significant differences were found between the oxygen consumption of animals from the two populations. The results of each experiment run on the Sapelo population were compared to the results of the most nearly similar experiment run on the Marshfield population. In 16 of the 18 comparable experiments no significant difference was found.

The oxygen consumption rates for animals from the two populations did not change significantly during the year. No significant differences were found between the rates determined for animals from the Sapelo population in November, 1961, and in March, 1962. Similarly no differences were found in the rates for animals from the Marshfield population in May and July, 1962.

The oxygen consumption of *Cyathura polita* increased at higher experimental temperatures. The mean oxygen consumption rate at 15° C. in the Sapelo popula-

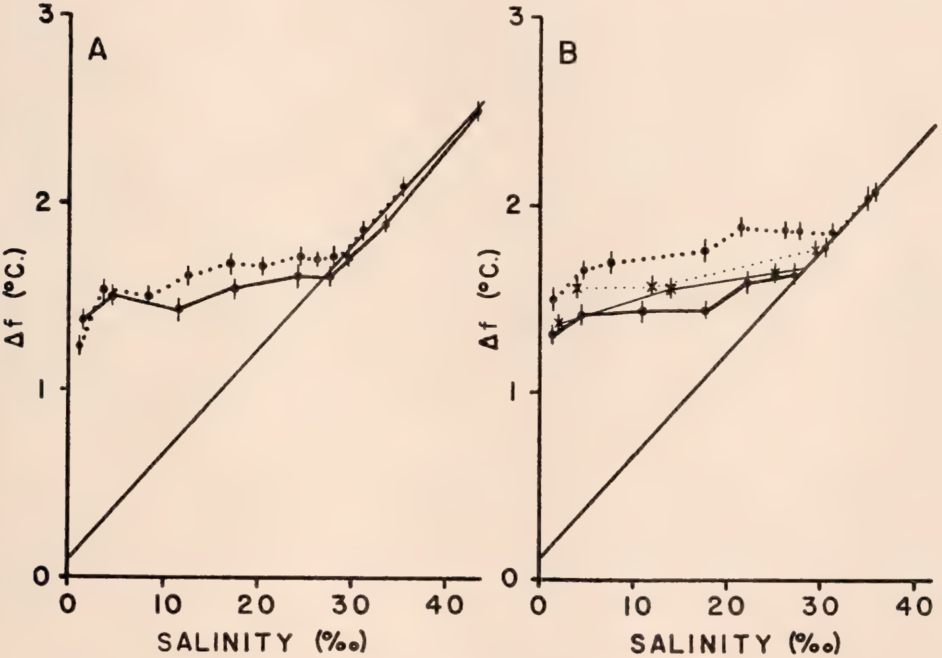

FIGURE 3. The relationship between the freezing point depression (Δ_f) of the body fluids of *Cyathura polita* and the salinity of the water to which the animals had been acclimated. Each point represents a mean value with vertical lines indicating plus and minus one standard error. The diagonal straight lines represent the depression of freezing point as a function of the salinity of sea water. Graph A shows the results of experiments run at 15–17° C.; graph B, the results of experiments run at 27° C. In both graphs heavy dotted lines represent results of experiments run on Sapelo Island animals in December, 1961, and heavy solid lines represent results of similar experiments on Marshfield animals in April, 1962. In graph B, the light dotted line connecting x's represents results of experiments run on Sapelo Island animals in March, 1962; the light solid line connecting x's represents results of experiments run on Marshfield animals in July, 1962.

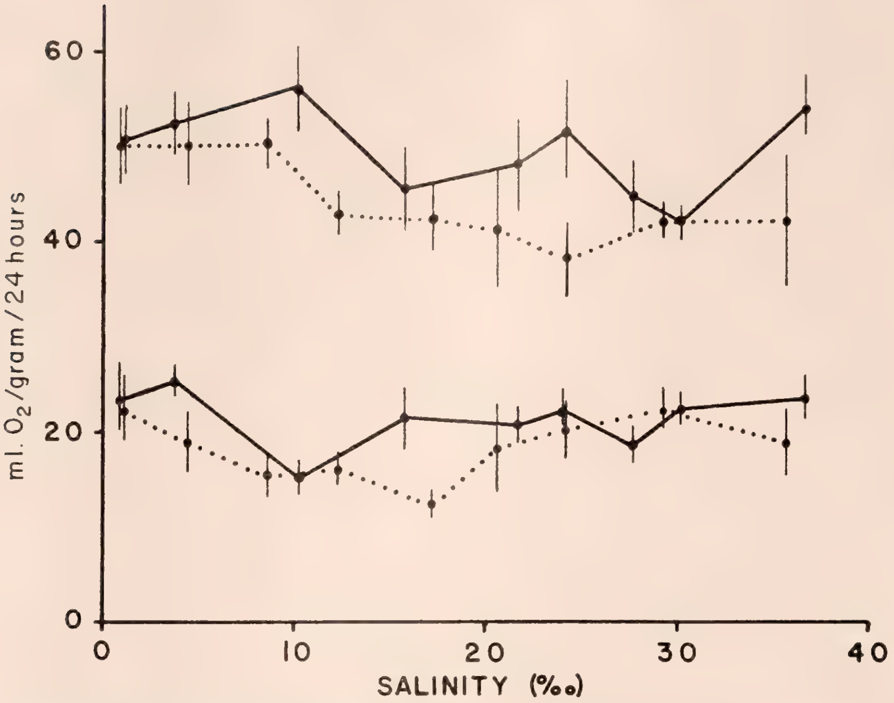

FIGURE 4. The relationship between the oxygen consumption rate of *Cyathura polita* and salinity. The oxygen consumption rate is expressed in milliliters of oxygen consumed per gram ash-free dry weight per 24 hours. Each point represents a mean value with vertical lines indicating plus and minus one standard error. Dotted lines represent results of experiments run on Sapelo Island animals in November, 1961, and solid lines represent results of experiments on Marshfield animals in May, 1962. The upper two lines show results of experiments run at 30° C., the lower two, results of experiments run at 15° C.

tion was 23.5 ml./gram ash-free dry weight/24 hours, and at 30° C. it was 44.6 ml./gram ash-free dry weight/24 hours. The corresponding rates for animals from the Marshfield population were 21.5 and 49.4 ml./gram ash-free dry weight/24 hours.

DISCUSSION

The habitats of the two populations of *Cyathura polita* appeared to be quite different, but closer analysis revealed that they were similar in many respects. Both had sandy sediments, well aerated water and wide fluctuations in salinity and temperature.

Ecological characteristics which were common to both populations were the irregular grouping (Cole, 1946), seasonal changes in sex ratio, and the numerical importance of cyathurans in the faunal associations. The grouped distribution pattern was more fully developed in the Sapelo population, possibly reflecting a subtle interaction of environmental conditions which made some areas of the beach particularly favorable for *Cyathura polita* (Delamare Deboutteville, 1960). The seasonal sex ratio change, currently reported and previously noted by Burbanck (1962), may be a characteristic of the genus since it has been observed both in *C. polita* and in *C. carinata* (Cléret, 1960).

C. polita is an important constituent of many estuarine benthic associations along the Atlantic coast of the United States (Burbanck, 1959), has been credited with being the dominant organism in certain specialized situations (Jones and Burbanck, 1959), and was one of the three most numerous species at both Marshfield and Sapelo. Yet the numerically important species associated with *C. polita* at one location, such as *Nassa obsoleta* and *Uca pugilator* at Sapelo, are not necessarily the abundant species at another cyathuran habitat (see Table II). Thus, although the fact that 20 of the 21 species found with *C. polita* at Marshfield and Sapelo had previously been reported as associates would suggest that this estuarine isopod is a member of an ecological community, the lack of consistent associates makes it impossible to compile a list of characteristic species for such a community. *Cyathura polita* seems capable of living in a wider range of habitat niches than other estuarine forms and so may be a member of many communities. For example, the one species not previously reported as an associate of *C. polita* but present at Marshfield was the fresh-water mussel, *Anodonta implicata.* Many of the streams which Burbanck (1962) has studied on Cape Cod have their upper and lower reaches separated by dams and tidal gates, but the uninterrupted flow of the South River probably allowed cyathurans to migrate into the area inhabited by *A. implicata.*

An ecological difference between the two populations was the size of the animals. Those from Marshfield were significantly larger than those from Sapelo. This difference seems to be characteristic of cyathurans and may be similar to the inverse size-temperature relationship found in the fresh-water copepod, *Cyclops vernalis* (Coker, 1934). However, the difference might be a reflection of the unusual habitat of the Sapelo population.

The body fluids of cyathurans from both populations were maintained hypertonic to their environment at low and medium salinities, but became isosmotic with the environment at salinities greater than 28‰. This type of osmoregulatory behavior is common among fresh- and brackish-water organisms (Prosser and Brown, 1961; Lockwood, 1962). It has been described in the shore crabs, *Carcinus maenas* (Duval, 1925; Schlieper, 1929), *Hemigrapsus nudus, H. oregonensis, Rithropanopeus harisii* (Jones, 1941); the European river crab, *Potomon edulis* (Duval, 1925); and the isopods, *Mesidotea entomon* (Bogucki, 1932; Lockwood and Croghan, 1957), *Ligia oceanica* (Parry, 1953), and *Asellus aquaticus* (Lockwood, 1959). The results of the present study show that *C. polita* is able to maintain a constant body fluid concentration over a wider range of salinities than many other isopods.

Seasonal variation in body fluid concentration has been found in a number of Crustacea. Lockwood (1960) has studied this relationship on a monthly basis in *Asellus aquaticus* and has found both a gradual variation with season and occasional sharp fluctuations from month to month. Seasonal variations were found in the body fluid concentration levels of *Cyathura polita.*

It is difficult to compare the oxygen consumption rates of different organisms because these rates are affected by many factors, including "activity, temperature, nutrition, body size, stage in the life-cycle, season, and the time of day, as well as previous oxygen and genetic background" (Prosser and Brown, 1961, p. 157). However, results of different experiments can be compared to illustrate basic relationships (Wolvekamp and Waterman, 1960). Thus the oxygen consumption of *Cyathura polita* resembles that of the isopods, *Porcellio scaber* (Reinders, 1933), *Asellus aquaticus* (Fox *et al.*, 1935), *Armadillidium pallasii* (Müller, 1943), *Oniscus asellus* (Edwards, 1946; Edney and Spencer, 1955), *Armadillidium vulgare* (Edney and Spencer, 1955), *Ligia oceanica* (Ellenby, 1951; Edney and Spencer, 1955), and *Idothea baltica* (Stoicovici and Rosca, 1958). It is lower than that described for such active forms as the amphipod, *Talitrus sylvaticus* (Clark, 1955) and the copepods, *Cyclops leuckarti*, *C. strenuus*, and *Diaptomus graciloides* (Shtcherbakov, 1935), but it is higher than that of such relatively large forms as *Homarus americanus* (Bosworth *et al.*, 1936) and *Uca pugnax* (Brown *et al.*, 1954) or such sedentary forms as *Libinia dubia* (Vernberg, 1956).

The oxygen consumption of *Cyathura polita* remains relatively uniform over a salinity range from 1 to 37‰ (Fig. 4). This relationship is similar to that which has been described in *Artemia salina* females (Gilchrist, 1956), in *Eriocheir sinensis* (Schwabe, 1933), and in the snail, *Theodoxus fluviatilis* (Bielawski, 1960).

The two *Cyathura polita* populations studied seem to be similar physiologically. Animals from both populations had the same type of osmoregulatory behavior and statistically similar oxygen consumption rates. In addition all experimental animals showed a direct relationship between body fluid concentration and temperature, and had oxygen consumption rates which were similar over a salinity range from 1 to 37‰. These similarities are especially striking since the two populations were studied at different seasons. Differences between the populations might be discovered by year-round study of each and by testing reciprocally transplanted animals, but the results of the present study seem to indicate that *Cyathura polita* is a relatively homogeneous species.

Species in which physiological races have developed are characterized by several kinds of variability. Burbanck and Burbanck (1961) have suggested that variability similar to that described in the ecological races of *Potentilla glandulosa* (Clausen and Hiesey, 1958) can be recognized in *Cyathura polita*. Burbanck and Burbanck (1961) studied dorsal chromatophore patterns and have shown that some variation occurs within local populations, that populations living under similar environmental conditions may have slightly different patterns, and that northern and southern populations have distinctly different patterns.

The apparent conflict between the results of the present study and that of Burbanck and Burbanck (1961) can be resolved, however. Clausen (1958) has pointed out that characters which fit a species to its environment are usually homogeneous in comparison with characters which do not. The physiological characters measured in this study were chosen because of their importance in fitting *Cyathura polita* to the estuarine environment. Therefore it is understandable that populations of *C. polita* might have different dorsal chromatophore patterns, but similar osmoregulatory behavior and oxygen consumption rates.

The physiological similarity of the two widely separated *Cyathura* populations may be typical of estuarine organisms. Clausen (1954, p. 477) has suggested that, "natural selection has through geologic ages sifted the naturally occurring (genetic) variability into workable and balanced combinations of genes and gene blocks that fit certain ecologic niches." Since estuarine niches are characterized by fluctuating environmental conditions, genetic variations which might reduce the tolerance of estuarine organisms to environmental fluctuations would tend to be eliminated by natural selection. This elimination would lead to relatively stable genetic structures. Perhaps the observed evolutionary conservatism of estuarine forms (Hedgpeth, 1957) can be explained not only by the relative permanence of the estuarine environment, but also by the limitations which the estuarine environment puts upon genetic variability. It would be interesting to analyze other estuarine species with wide distributions to see if they show the same physiological homogeneity as does *Cyathura polita*.

The authors are indebted to the following for their aid in the identification of specimens: Dr. E. L. Bousfield, Amphipoda; Dr. Marian Pettibone, Polychaeta; and Dr. William J. Clench, Pelecypoda.

SUMMARY

1. Aspects of the physiology and ecology of two populations of the estuarine isopod *Cyathura polita* have been studied. One of the populations was located in a beach on Sapelo Island, Georgia; the other, in an estuary in Marshfield, Massachusetts. The ecological aspects of the study included habitat observations; analyses of the density, animal size, dispersion and sex ratio of the populations; and investigation of the faunal associations. The physiological aspects included studies of osmoregulatory behavior and oxygen consumption rates.

2. Ecologically, the two populations were similar in a number of respects. They were both found in habitats which had sandy sediments, well aerated water, and wide fluctuations of temperature and salinity. The Sapelo Island population was less dense, and was made up of smaller animals than the Marshfield population, but they both had clumped dispersion patterns and sex ratios which changed seasonally. The faunal associations were limited, and although *Cyathura polita*, and *Scoloplos fragilis*, were the only species found in both areas, others were closely related. *C. polita* was one of the most common animals in both associations.

3. The physiological studies showed that cyathurans from the two populations have similar osmoregulatory behavior and oxygen consumption rates. *Cyathura polita* maintains a body fluid concentration which is hypertonic to its environment at salinities between 1 and 28‰, but which becomes isosmotic with the environment at salinities between 28 and 42‰. The oxygen consumption rate of *Cyathura polita* is similar to that of other isopods. The rate increases at higher temperatures, but does not change significantly over a salinity range from 1 to 37‰.

4. The physiological similarity of the two populations seems to indicate that distinct physiological races have not developed within the species. This physiological homogeneity may be an example of the evolutionary conservatism of estuarine organisms.

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DEVELOPMENT AND FUNCTION OF NEUROSECRETORY SITES IN THE EYESTALKS OF LARVAL PALAEMONETES (DECAPODA: NATANTIA)[1]

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To comparative endocrinologists, *Palaemonetes* is a classic experimental animal. Five species inhabit the eastern United States. *Palaemonetes vulgaris* (Say), *P. pugio* Holthuis and *P. intermedius* Holthuis are marine or estuarine forms. *Palaemonetes kadiakensis* Rathbun and *P. paludosus* (Gibbes) inhabit fresh water. The larval development of all five species has been described (Broad, 1957; Broad and Hubschman, 1960, 1962; Faxon, 1879; and Dobkin, personal communication).

Our knowledge of the development of neurosecretory systems in larval crustaceans is meager and based essentially upon Pyle's (1943) work on *Homarus* and *Pinnotheres*. Matsumoto (1958) worked on newly hatched crabs; the species he studied, however (*Potamon dehaani*), like many fresh-water decapods, hatches in the form of a small adult, having passed within the egg those stages corresponding to the larval stages of other species. Dahl (1957) described the embryology of the X-organ in *Crangon allmanni*. His paper unfortunately contained no figures and no information on activity of the cells observed. Broch (1960) studied the effects of tissue extracts on isolated chromatophores of *Palaemonetes* zoeae. Costlow (1961, 1962) reported on the effects of *Sesarma* zoeal eyestalk extracts on adult chromatophores and the effects of removal on the megalops of *Callinectes*.

Experimental and histological data concerning the development of neurosecretory sites and functions in the eyestalks of larval *Palaemonetes* are presented here.

METHODS

Rearing of larvae

The procedure for rearing was essentially the same for both fresh-water and marine species, and followed that outlined by Broad (1957). *Palaemonetes kadiakensis* was collected in and around the Sandusky Bay region of Western Lake Erie during the summer of 1960. This portion of the work was carried out at the Franz Theodore Stone Laboratory at Put-in-Bay, Ohio. The shrimp were kept in aquaria containing Lake Erie water and fed macerated parts of several local fishes, usually whole emerald shiner, *Notropis atherinoides*. Ovigerous females were examined daily. When embryos reached a late stage of development, the female was isolated in a culture dish until the eggs hatched. Upon hatching, the entire brood was placed in a clean dish and fed day-old *Artemia* nauplii. The larvae were fed and the water changed daily until they reached a desired stage of development, at which time they were fixed. The larvae of some broods were fixed all at the same time, while from other broods, samples were removed throughout the developmental period.

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Several females produced eggs in the laboratory. By using some of these eggs, a series of embryos was fixed for later sectioning. Eggs were fixed at two-hour intervals during the first day and 12-hour intervals thereafter until hatching. Individuals of one group were fixed at 6-hour intervals during the last three days of embryonic development.

Late embryos, larvae, and post-larvae of the other fresh-water species, *P. paludosus*, fixed in alcoholic Bouin's solution, were supplied by Mr. Sheldon Dobkin of the Marine Laboratory, University of Miami, Florida.

Rearing and experimental work on larval stages of the marine species were carried out at the Duke University Marine Laboratory, Beaufort, North Carolina. Adult *P. vulgaris*, *P. pugio*, and *P. intermedius* were collected in the tidal areas around Beaufort throughout the summer of 1961. The shrimp were maintained in sea-water aquaria on a diet of the soft parts of the mud snail, *Nassarius obsoletus*, the mussel, *Modiolus demissus*, and the oyster *Crassostrea virginica*. Females with eggs in late stages of development were isolated in individual bowls of sea water. After hatching, some larvae were reared in mass culture as above, while those used in experiments were placed in individual bowls (3″ Carolina stacking culture dishes), and complete molting and developmental histories were kept. All larvae were fed *Artemia* nauplii. The sea water in which larvae were reared was adjusted to 30 ± 1‰ salinity by the addition of demineralized water, and was changed every day. At the same time, the larvae were examined and a new supply of food was introduced.

There are five to eight molts prior to metamorphosis in *P. kadiakensis*, from six to eight in *P. intermedius*, and seven to eleven premetamorphic molts in *P. vulgaris* and *P. pugio* (Broad and Hubschman, 1962). To avoid confusion in this paper, all references to larval forms (Roman numerals) have been made in terms of the six described for *P. kadiakensis* (Broad and Hubschman, 1963).

Histological methods

All larval stages of *P. vulgaris*, *P. pugio*, *P. intermedius*, *P. kadiakensis*, and *P. paludosis* were sectioned. Preliminary to the study of larval tissues, the eyestalks of adult *P. vulgaris*, *P. pugio*, *P. intermedius* and *P. kadiakensis* were studied, establishing a basis for the interpretation of the developmental processes.

The fixing reagents used were 5% formalin, formalin-alcohol-acetic acid (FAA or AFA), Zenker's fluid, Helly's fluid, and Smith's alcoholic Bouin's solution (Guyer, 1953). Fixation time varied from 8 to 24 hours, depending upon the tissue and reagent. Separate eyestalks were washed in crustacean saline to remove blood and then immersed in alcoholic Bouin's solution. Freshly dissected eyestalks were studied by the methylene blue technique suggested by Bliss and Welsh (1952).

All tissues were sectioned by the paraffin method. Preparatory to dehydration and embedding, the larval cuticle was broken to insure penetration of the embedding medium. A tangential cut was made through the ommatidia at the distal end of the eyestalk. Some thoracic appendages and the posterior portion of the abdomen were also removed. A similar tangential cut was made through the ommatidia of separate adult eyestalks. These operations were performed under a stereoscopic microscope at 40 ×.

Smith's alcoholic Bouin's fixative was found to produce the most satisfactory results. Problems of overfixation were not encountered, and the cuticle was not rendered brittle, as it was following Zenker or Helly fixation. The latter reagents also produced background coloration with the stains used. Fixation in formalin or AFA did not always provide good differentiation, but this was corrected by mordanting the sections in Bouin's fluid during the hydration process.

The most frequently used staining procedure was a modification of an azocarmine-aniline blue-orange-G process suggested by Gomori (1939, 1946). This modification has been described previously (Hubschman, 1962) and will be referred to from here on as azan.

Sections intended for general histological study were also stained in Mallory's triple connective tissue stain or with acid fuchsin followed by the counterstain of the azan process. Stains that are semiselective for neurosecretory material have been proposed by a number of workers. Two of these that have been widely used for crustacean tissues were employed here. The first, Gomori's (1941) chrome-hematoxylin phloxin, will be referred to hereafter as CHP. The other, Dawson's (1953) modification of Gomori's (1950) aldehyde fuchsin, will be designated PAF.

Destalking experiments

Experiments involving eyestalk removal from adult shrimp were primary in the sequence of events that originally established these structures as control centers of metabolic activity. To evaluate the role of eyestalks in larval development, a series of experiments was conducted with zoeal forms of *Palaemonetes pugio*. A group of sibling larvae (usually an entire brood) was separated into experimental and control groups. Each shrimp was placed in an individual bowl of sea water and assigned a number. Rearing methods were those described in the previous section. Complete molting and developmental records were kept for all larvae. Each day the shrimp were removed with a large-bore pipette and examined under a stereoscopic microscope. Any change in developmental form was recorded and the dish examined for the presence of exuviae.

Eyestalks were removed with a fine blade honed from a #2 insect pin mounted in a wooden handle. Three or four hours before the operation, the larvae were cooled to 10° C. The eyestalks were then severed at the base and the animals returned to room temperature over a period of two hours. Later, since it was found that the operation performed at room temperature resulted in no higher mortality than resulted after the cooling process, that step was eliminated. About 75% of the larvae survived the operation.

Two types of control animals were used. The first was unaltered, normal larvae. The antennae of the second type were removed to allow for possible differences in molting or development resulting from operative stress. Removal of the antennule, though an easy operation, resulted in excessive bleeding, owing to the relatively large size of the base of that appendage in early zoeal forms. Consequently, the antennal scale and flagellum of the operated control animals were removed by cutting through the basal segment (either basipodite or coxopodite). Except for the operation, all larvae were treated in the same manner. The first zoea hatches from the egg with eyes enclosed in the protozoeal carapace and cannot be treated as a stalk-eyed form. Experiments were performed on all later larval stages.

Laboratory conditions did not permit precise control over temperature during the experiments. The room used was air-conditioned, but high outside temperatures and equipment operating in other parts of the laboratory placed a burden on the system. Thus, during the period of the experiments, the mean daily temperature fluctuated between 23.7° and 27.0° C. with an average of about 25.5° C. The average daily fluctuation in temperature was 3.47° C., but twice was 6.0° C.

RESULTS

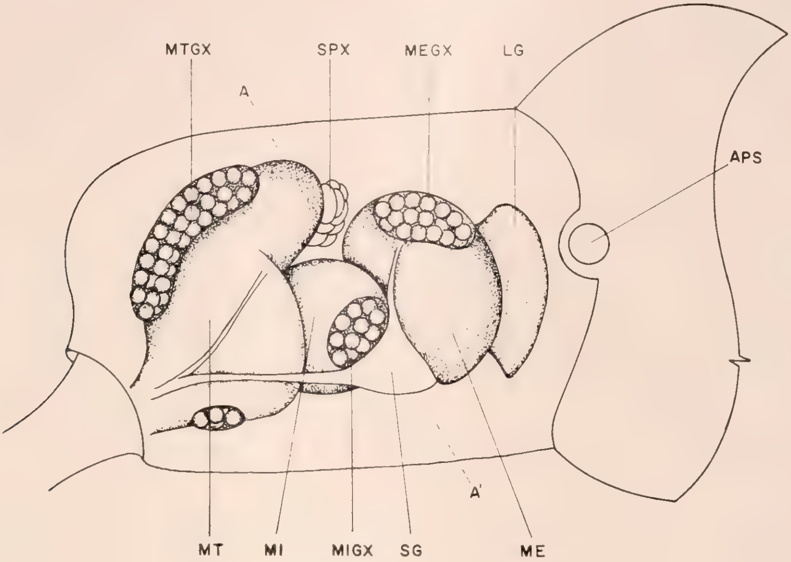
Adult eyestalks

As a basis for comparative study, eyestalks of adult shrimp were examined on the living animal, as freshly dissected eyestalks, and in serial histological section. The distribution of neurosecretory cell groups was found to agree closely with that described by previous workers. The eyestalk organs of *P. vulgaris* were pictured by Hanström (1939) in his survey of crustacean central nervous systems. The histology of the eyestalk of *Palaemonetes*, as well as that of *Uca*, was presented in some detail by Milburn (1958). An account of the anatomical aspects of neurosecretory systems in adult *Palaemonetes* and *Uca* is apparently in preparation at this time (Milburn, personal communication).

The eyestalks of *P. vulgaris*, *P. pugio*, *P. intermedius* and *P. kadiakensis* were found to be essentially alike. Figure 1 is a diagrammatic sketch of the eyestalk of *P. kadiakensis*. In keeping with the suggestion of Carlisle (1959), the terminology of Russell is used in describing the relationships of the structures discussed here. The sinus gland, shown to be composed of nerve fiber endings (Bliss and Welsh, 1952), is located on the dorsal, abaxial surface of the ganglionic mass, in a crevice between the *medulla interna* and the *medulla externa*. It surrounds the junction of the inner and outer blood sinuses. The X-organ of Hanström, the *pars distalis* or the sensory pore X-organ (referred to hereafter as SPX), occupies a position opposite the sinus gland (Fig. 2). It is located between the adaxial, ventral surface of the *medulla terminalis* and the cuticle. The sensory pore proper is positioned ventrally in the adult eyestalk. There are secretory cell groups associated with each of the ganglionic masses. Beginning at the proximal end of the eyestalk (Fig. 1) they are: the *pars ganglionaris* or *medulla terminalis* ganglionic X-organ (MTGX); the *medulla interna* ganglionic X-organ (MIGX); and the *medulla externa* ganglionic X-organ (MEGX).

Two important additional details were observed in fixed and stained preparations. The first was the existence of an extremely large, solitary, monopolar, secretory neuron. This cell is located on the abaxial surface of the *medulla interna* and closely associated with, but seemingly not contributory to, the sinus gland tract (Fig. 3). Its axon penetrates the fiber mass of the *medulla terminalis*. This giant neuron has a diameter of approximately 40 μ. It has a centrally placed nucleus (diameter 19 μ) containing two prominent nucleoli. The cell usually stains blue with azan and bronze to purple with PAF. This cell is an important feature of the larval eyestalk and will be discussed later.

The second histological feature that can be observed consistently is the vacuolar nature of the lobes of the SPX (Fig. 4). Hanström (1939) noted that the vacuolar portions appeared empty (except for secretory droplets) in preparations fixed in Bouin's solution, but they were filled with a stainable substance following Zenker fixation. (At times there were large accumulations of droplets in the vacuole



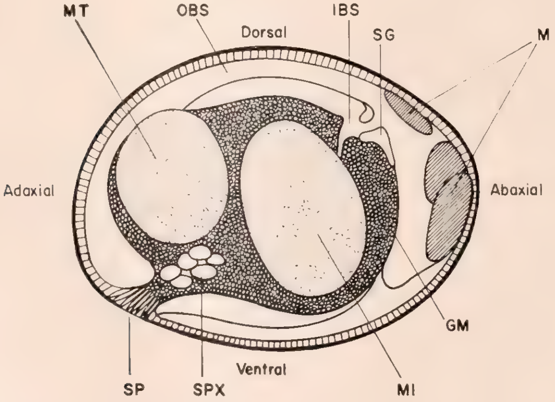
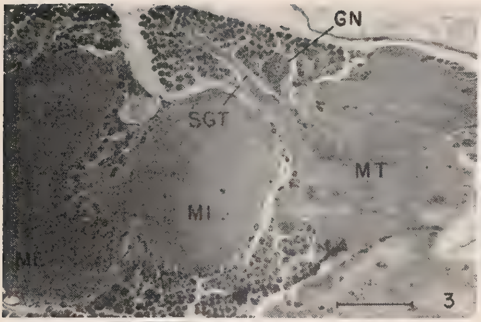
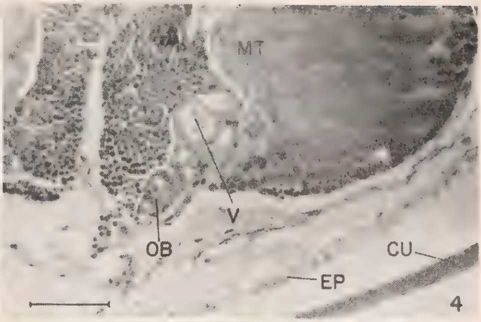
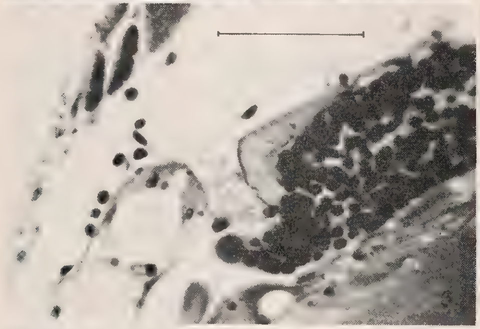


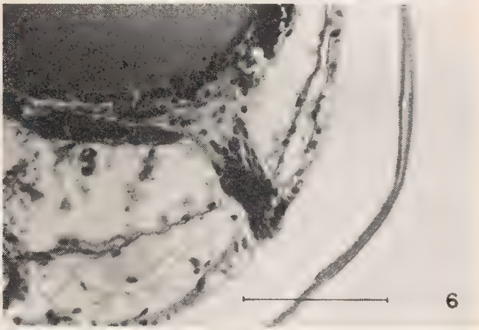
FIGURE 1. Dorsal view of the right eyestalk of adult *Palaemonetes kadiakensis.* The muscles, connective tissue, and most ganglionic nuclei have been omitted for clarity. APS, accessory pigment spot; LG, *lamina ganglionaris;* ME, *medulla externa;* MEGX, *medulla externa* ganglionic X-organ; MI, *medulla interna;* MIGX, *medulla interna* ganglionic X-organ; MT, *medulla terminalis;* MTGX, *medulla terminalis* ganglionic X-organ; SG, sinus gland; SPX, sensory pore X-organ.

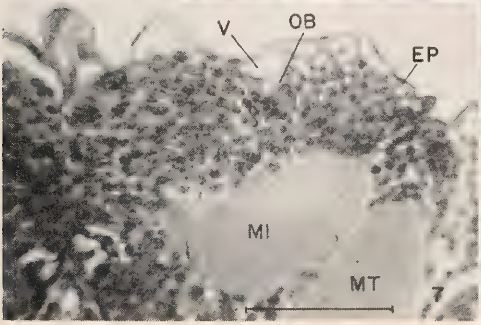
FIGURE 2. Diagrammatic section through A-A' of Figure 1. GM, nuclei of ganglionic mass; IBS, inner blood sinus; M, muscle; MI, *medulla interna;* MT, *medulla terminalis;* OBS, outer blood sinus; SG, sinus gland; SP, sensory pore; SPX, sensory pore X-organ.











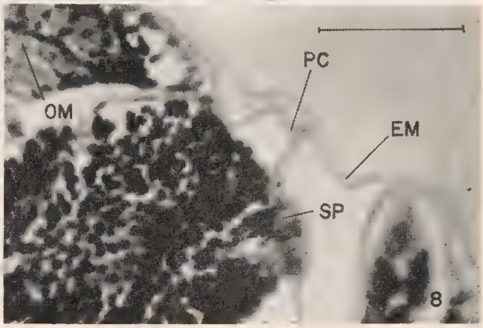


FIGURE 3. Frontal section through the eyestalk lobes of adult *Palaemonetes pugio* (azan stain). ME, *medulla externa;* MI, *medulla interna;* MT, *medulla terminalis;* GN, giant neuron; SGT, sinus gland tract. Scale represents 100 μ.

FIGURE 4. Section through sensory pore X-organ of adult *Palaemonetes pugio* (azan stain). CU, cuticle; EP, epidermal layer; MT, *medulla terminalis;* OB, onion bodies; V, vacuole with secretory droplets. Scale represents 100 μ.

FIGURE 5. Sinus gland of adult *Palaemonetes kadiakensis.* Compare with Figure 2 for anatomical relations (azan stain). Scale, 100 μ.

FIGURE 6. Sensory pore cells of adult *Palaemonetes kadiakensis.* Compare with Figure 2 (azan stain). Scale, 100 μ.

FIGURE 7. Section through eyestalk of *Palaemonetes kadiakensis* embryo (azan stain). EP, epidermal layer; MI, *medulla interna;* MT, *medulla terminalis;* OB, *onion bodies;* V, vacuole of SPX. Scale, 100 μ.

FIGURE 8. Section through dorsal surface of embryonic eyestalk (azan stain). EM, egg membranes; PC, protozoeal cuticle; OM, developing ommatidia; SP, ganglionic cells elongating in the formation of the SPX. Scale, 100 μ.

regardless of fixation.) Hanström considered these structures to be secretory and attributed differences in appearance to stages in secretory production. Since then, the so-called onion bodies associated with these vacuoles have been shown to be nerve fiber terminations (Carlisle and Knowles, 1959).

Larval eyestalks

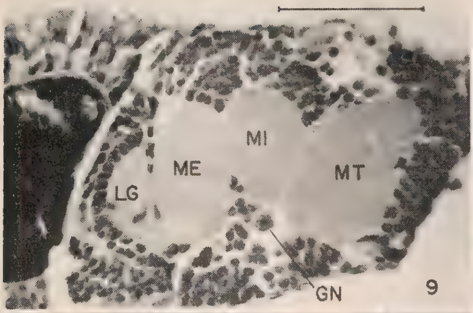
The histogenesis of neurosecretory structures was essentially the same in *P. kadiakensis*, *P. pugio*, *P. vulgaris* and *P. intermedius*. *Palaemonetes paludosus*, which has an abbreviated larval development (Dobkin, personal communication), passed through the same sequence of changes in a shorter period of time.

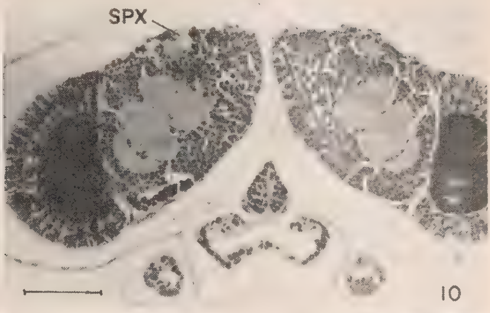
The incubation period of *P. kadiakensis* can be expected to range between 24 and 28 days (Broad and Hubschman, 1963). The topography of the definitive eyestalk is recognizable from about the tenth day. Prior to this, the eyestalk region is composed of undifferentiated neuroblasts. With differentiation, the nerve cells have characteristic brilliant red nuclei (following azan) and very sparse cytoplasm. The cytoplasm of these cells is confined almost entirely to the axons stretching far from the cell body. The neuropile appears pale purple. Undifferentiated neuroblasts contain cytoplasm that takes up the orange-G component of azan.

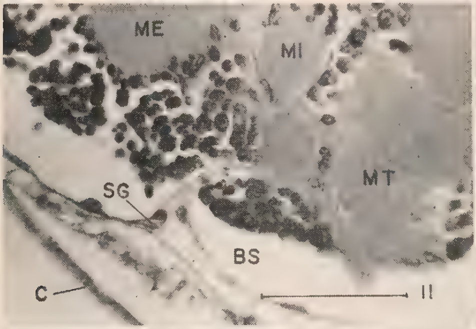
About 5 or 6 days before the hatching, the structures contributing to the future SPX can be seen. The most obvious feature of this complex is a single cavity or vacuole closely applied to the outer surface of the eyestalk (Fig. 7). This cavity contains a very fine filamentous substance, strands of which are near the limit of resolution of the light microscope. This has the appearance of connective tissue and stains blue with azan. The thickened wall of the cavity is in direct contact with the epidermal layer at this time. Onion bodies can be seen forming in nests of ganglionic cells. These are always more axial than the vacuole and do not contact the surface of the eyestalk. The onion bodies stain blue, but not as strongly as does the vacuolar portion. The vacuole-onion body complex (SPX) is surrounded by undifferentiated neuroblasts and occupies a position at the dorsal surface of the eyestalk. In some preparations a fine connection can be traced from the *medulla terminalis* to the SPX. It may be that a nerve fiber migrates some distance from the neuropile and then its terminal portion differentiates under the influence of specific cells. This gives the appearance of the nests mentioned earlier. It could not be determined whether or not the onion body was actually within the cytoplasm of the nest cell.

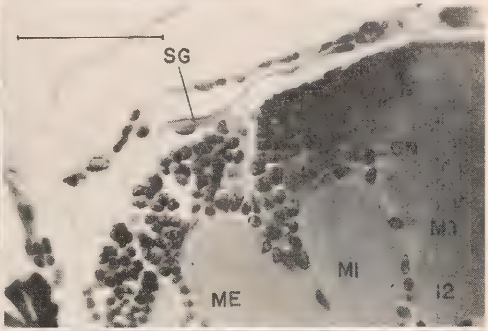
Just before the hatching, the onion bodies become more conspicuous. At this time they stain more deeply with aniline blue, but this may be simply because they are larger and more dense. Nerve cells on the surface of the ganglionic mass have begun to assume the elongated shape of sensory cells (Fig. 8). The sheath of the vacuole is now clearly cellular. Several onion bodies appear in the lower (axial) portion of the vacuole. At this time there seem to be three membranes present: one around the vacuole, one around the onion bodies, and an outer one covering both.

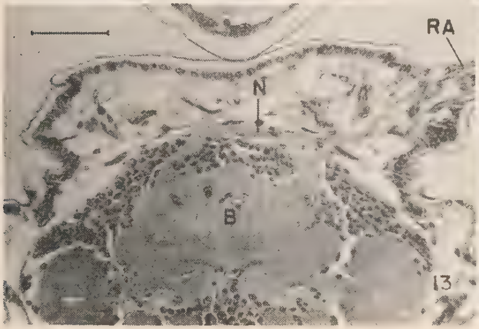
At hatching (Form 1) the vacuole is approximately 15 μ in diameter. Individual onion bodies are up to 6 by 8 μ in diameter. Mitotic figures are numerous in the ganglionic mass. A presumptive neurosecretory cell appears in the crevice between the *medulla terminalis* and the *medulla interna* (Fig. 9). This is the











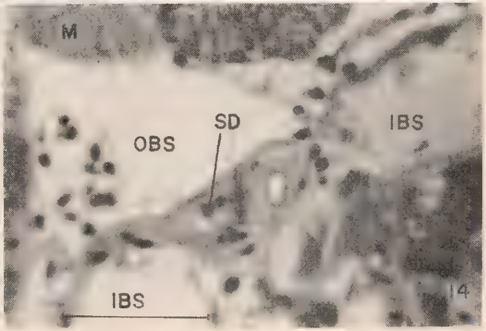


FIGURE 9. Section through the eyestalk of the first larval stage (Form I) of *Palaemonetes intermedius* (azan stain). GN, giant neuron in its primary (ventral) position; LG, *lamina ganglionaris;* ME, *medulla externa;* MI, *medulla interna;* MT, *medulla terminalis.* Scale, 100 μ.

FIGURE 10. Section through the anterior end of the first larval stage (Form I) of *Palaemonetes kadiakensis* (azan stain). SPX, sensory pore X-organ, (vacuole appears in eyestalk at left, onion body clusters can be seen in both eyestalks). Scale, 100 μ.

FIGURE 11. Sinus gland forming in the fifth larval stage (Form V) of *Palaemonetes pugio* (PAF stain). C, cuticle; BS, blood sinus; ME, *medulla externa;* MI, *medulla interna;* MT, *medulla terminalis;* SG, sinus gland. Scale, 100 μ.

FIGURE 12. Compact sinus gland of Form VII (first post-larva) of *Palaemonetes pugio* (PAF Stain). GN, giant neuron; ME, *medulla externa;* MI, *medulla interna;* MT, *medulla terminalis;* SG, sinus gland, 100 μ.

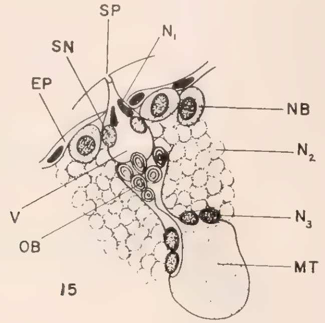
FIGURE 13. Section through the anterior end of *Palaemonetes pugio* from which eyestalks had been removed during larval development (azan stain). B, brain; N, nerve fiber tract crossing over and merging with nerve supply of regenerated appendage; RA, regenerated appendage. Scale, 100 μ.

FIGURE 14. Sinus gland of juvenile *Palaemonetes pugio* with accumulation of secretory material (azan stain). IBS, inner blood sinus; OBS, outer blood sinus; M, muscle; SD, secretory droplets; SG, sinus gland. Scale, 100 μ.

giant cell of the MIGX. It seems to differentiate from a ganglionic cell rather than from a typical neuroblast. This giant cell changes in staining reaction during the first larval stage. At the time of hatching it stains orange (as do the neuroblasts) but, by the first larval molt, the cell appears blue after azan and changes from pale to deep purple with PAF.

During the second larval stage (Form II), the onion bodies continue to increase in size and number. The SPX is by now a definite organ. It is no longer in contact with the surface of the eyestalk, but is still dorsal in position (Fig. 10). At this time, very small secretory droplets can be seen in the vacuole. These droplets are barely visible and appear only as dark specks after all stains.

By the time that the animal has reached Form III, the onion bodies become collectively larger than the vacuole. Individual bodies may range up to 11 by 16 μ in size, while the whole organ may be 45 μ in diameter. Neuroblasts around the



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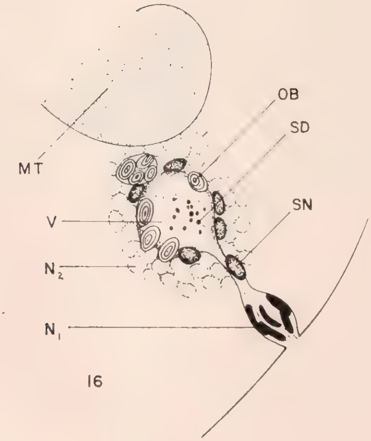

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FIGURE 15. Diagram of sensory pore X-organ in the third larval form of *Palaemonetes*. EP, epidermal cell; MT, *medulla terminalis;* N$_1$, nucleus of sensory pore cell; N$_2$, nucleus of ganglion cell; N$_3$, nucleus of cell contributing a process to the SPX; OB, onion body; NB, neuroblast; SN, nucleus of sheath cell; SP, sensory pore; V, vacuole of SPX.

FIGURE 16. Diagram of sensory pore X-organ of post-larval *Palaemonetes*. MT, *medulla terminalis;* N$_1$, nucleus of sensory pore cell; N$_2$, nucleus of ganglionic cell; OB, onion body; SD, secretory droplet; SN, nucleus of sheath cell; V, vacuole.

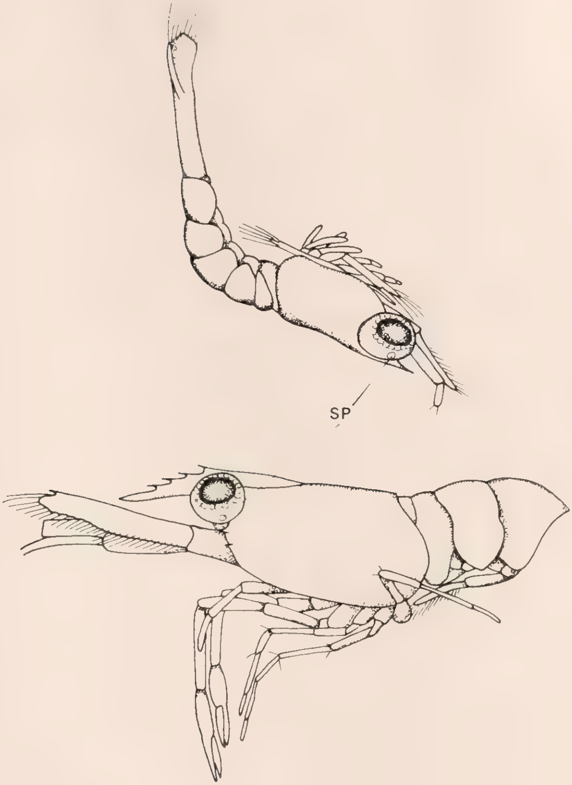

FIGURE 17. Diagram showing the attitude of larval (A) and post-larval (B) shrimp with reference to the relative position of the sensory pore X-organ.

SPX continue to divide. The vacuole is approximately 18 μ in diameter and contains more droplets. These droplets stain orange with azan and deep purple with PAF. A layer of ganglionic nuclei has now formed between the SPX and the surface of the eyestalk. Slightly elongated, epidermal, sensory cells project in a cone to the pore at the surface. The pore is approximately 5 μ wide and 7 μ deep (Fig. 15).

The SPX continues to be displaced, by differential growth, from its peripheral location to a site deeper in the ganglionic mass. Throughout larval life, the onion bodies increase in number while a single cavity remains.

Eventually, the onion bodies come to lie between the vacuole and the surface of the eyestalk, as they do in the adult. The accumulation of secretory droplets becomes continually greater. There is, however, no evidence of secretory activity by cells immediately surrounding the SPX.

By the fifth larval stage, but not earlier, the sinus gland can be recognized. While not a functional complex as yet, an accumulation of nerve endings can be seen at the site of the future junction of the external and internal blood sinuses (Fig. 11). The nerve fibers, mostly from the *medulla terminalis*, form a delicate fan extending over the sinus area. The giant cell is in its definitive position and a number of presumptive neurosecretory cells occupy the site of the future MIGX. This area is ventrally located in the larval eyestalk.

The vacuole is a constant feature of the SPX complex from the fifth day before the hatch to the definitive adult organ. The adult conformation of the SPX can be recognized in the early post-larval shrimp (Fig. 16). At metamorphosis, the eyestalk begins to rotate on its longitudinal axis. The result is that, by the third post-larval molt, the SPX is ventral. This is just the opposite of the embryonic and larval position on the dorsal surface of the ganglionic mass. This rotation in effect results in the maintenance of the orientation of the sensory pore with the substrate, while the rest of the animal assumes the characteristic dorsal-side-up position of the adult (Fig. 17). In effect, the shrimp rotates around the fixed eyestalk.

At metamorphosis, the sinus gland develops its definitive shape (Fig. 12). No accumulation of secretory material could be detected during larval life. With the onset of post-larval existence (third or fourth post-larval molt), however, the sinus gland assumes functional importance. Examination of sections of destalked larvae revealed no build-up of stainable droplets in the brain stubs, as shown in adult crabs by Matsumoto (1958). Apparently the migration of neurosecretory substances from the brain and thoracic ganglion does not begin until post-larval life. The ganglionic X-organs of the adult eyestalks are not functional during larval development. Only after metamorphosis can the cells of the MTGX, MIGX, and MEGX be recognized histologically. With the procedures used, the secretory activity in these cells can be demonstrated for the first time in shrimp that have completed several post-larval molts.

Destalking experiment

In spite of the wealth of information available on decapod larval development, nothing is known of the mechanism of control of crustacean metamorphosis. Passano (1961) has suggested that an unknown hormonal control may exist, and has called our attention to the need for experiments involving interference with the normal process to support his hypothesis. The data reported here are derived from daily observations of 187 larval *P. pugio* (133 destalked larvae, 36 from which the antenna had been removed, and 18 intact controls). The mean intermolt period varies from 2.3 to 2.5 days, but the maximum duration of the intermolt does not exceed 5 days.

It seems obvious that the measurement of duration of molt cycle should have been made in some unit smaller than days. *Palaemonetes* larvae molt only at night, however (usually not before 8:00 or 9:00 PM), and observations more frequent than daily could add nothing of value to the data. The variate (*i.e.*, duration of molt cycle) is actually a discontinuous one and properly may be measured in days. No significant difference in duration of molt cycle could be observed between experimental and control animals.

Larvae from which eyestalks had been removed metamorphosed between 18 and 30 days after hatching (mean, 20.79 days). Operated control larvae metamorphosed between 20 and 25 days (mean, 22.88 days) and unaltered controls metamorphosed between 18 and 22 days (mean, 20.33 days). Analysis of variance fails to reveal that these differences are significant.

Growth may properly be separated from development. It was convenient, however, to consider the influence of eyestalk ablation on growth of the same groups of larvae studied above. As shown by experiments on adult *Cambarus* (Smith, 1940), *Uca* (Abramowitz and Abramowitz, 1940), and *Carcinus* (Carlisle, 1955), destalking results in increased size. Destalked larvae at metamorphosis had an average overall length (tip of rostrum to end of telson) of 10.1 mm. Control animals were approximately 7.1 mm. at the same stage of development. No differences in average length could be observed between altered and intact control animals.

Adult shrimp differ from their brachyuran relatives in the chromatophore response following eyestalk removal. Crabs exhibit an overall paling while shrimp darken following the operation. In adult *Palaemonetes* there is complete dispersal of red pigments following removal of the eyestalks (Perkins, 1928). While quantitative aspects of these phenomena were not studied here, it should be noted that eyestalk removal resulted in complete paling of the larval shrimp. The pale condition persisted through subsequent molts and developmental phases through metamorphosis. The immediate condition seemed to result from contraction of chromatophores, but possibly was accompanied by loss of pigment in later stages.

It has been observed previously that removal of eyestalks proximal to the optic ganglia will result in regeneration of an antenna in place of the missing eyestalk, in some adult decapods (Bliss, 1960). Regeneration of a short, setose appendage was commonly observed among the larvae destalked in these experiments. No external eyestalk or portion thereof was ever observed to regenerate. Larvae having antennae removed were found to replace this structure quite rapidly. Often the full antenna was present by the time of metamorphosis. In several instances the nerve supply to the regenerated antennule (in eyestalk position) arose from both sides of the brain. This crossing-over is shown in Figure 13.

Fingerman and Aoto (1960) have demonstrated in *Cambarellus* that, after severance of eyestalks, an accumulation of secretory products can be seen in the terminal portion of the remaining stub. This supports the findings of Matsumoto (1958) mentioned earlier. Adult shrimp (*P. kadiakensis*) were destalked to see if this build-up could be observed. The animals were held for 6 days, then fixed, sectioned, and stained with azan. An accumulation of red droplets was found in the nerve stubs of the destalked shrimp. The optic nerves of intact adults were free of droplets. It should be recalled that no accumulation of stainable material could be detected in the brain stubs of destalked larvae.

DISCUSSION

Dahl (1957), in his account of the embryology of the X-organs in *Crangon allmanni*, states that the first onion body lies directly beneath the integument and is surrounded by cells of the SPX. This is not the case in *Palaemonetes*. The most prominent feature of the SPX in this shrimp is the sac-like vacuolar portion, which, indeed, does contact the epidermal layer in early development (Fig. 7). The onion bodies are always more axial (*i.e.*, proximal to the neuropile) in position and never contact the integument. During larval development, however, these structures do change in relative position when the onion bodies bypass the vacuole, owing to differential growth. By the time the adult stage is reached, the vacuole lies between the onion body cluster and the neuropile (Fig. 4). While the vacuole may not be present in *Crangon*, this difference could be explained on the basis of similarity of staining properties of these structures. The developing vacuole and associated onion bodies both appear blue after azan, purple after PAF, and gray after CHP. A tangential section of the vacuole near the surface of the eyestalk could be misinterpreted as an onion body.

This sac-like SPX in the larval eyestalk appears more like the X-organ of the Mysidacea than it does the definitive form of adult *Palaemonetes*. In the mysids, *Eucopia* and *Boreomysis*, the X-organ is sac-like and situated close to the eye papilla (Hanström, 1939). In one (*Boreomysis*), the large vesicle is filled with intensely stained, winding threads that call to mind the filamentous inclusions of the larval SPX in *Palaemonetes*. The resemblance of many decapod larvae to the Mysidacea is superficial and probably reflects the pelagic environment (Snodgrass, 1956), but it is interesting that there is internal similarity, at least in one detail.

Other aspects of the developing SPX in *Palaemonetes* agree more closely with that in *Crangon*. Cells of the SPX, as well as the ganglionic X-organs, develop from neuroblasts of the ganglionic layer. As is the case in *Crangon*, these neuroblasts first assume the appearance of regular nerve cells, then mature into definitive types. Many of the cells of the SPX differentiate during embryonic development, and those of the ganglionic X-organs become recognizable only after metamorphosis. The first onion body appears 5 or 6 days before the hatch. As Dahl (1957) observed, this coincides with the appearance of eye pigments. There is no known significance to this occurrence, other than that it furnishes an outward clue to the state of development. This could be of aid to the experimental embryologist.

Carlisle (1953) has shown that onion bodies in *Lysmata* are expanded endings of nerve fibers emerging from the *medulla terminalis*. This appears to be the case in *Palaemonetes*. A fine connection can be traced from the *medulla terminalis* to the developing onion body. The end of this fiber appears to differentiate into an onion body while in close association with cells of the ganglionic mass. The developing body is surrounded by nuclei that seem to participate in the maturation process. This gives the appearance of the rosette or nest mentioned earlier. Since the nerve tract connecting the onion body cluster with the neuropile is not visible until the onion bodies form, the direction of growth cannot be stated with certainty. There is no reason, however, to believe that the nest cells send out the process in the direction of the *medulla terminalis*.

The remainder of the SPX, including the terminal sensory pore portion, is derived from cells of the nerve ganglion. There is no evidence to support the view that the sensory pore complex is derived from epidermal cells. Late in embryonic development, the sensory portion develops from ganglion cells that elongate within the ganglionic mass (Fig. 8).

Throughout embryonic development and larval life, the cells of the future ganglionic X-organs fail to show any activity with CHP or PAF. The single exception to this is the giant cell located peripherally to the MIGX, close to the sinus gland tract. This cell apparently becomes active early in larval life. During the first phase of free-living existence (Form I), this cell stains deeply with aniline blue and appears purple with PAF (Fig. 9). Since this is the only cell that seems to be active during larval life, and its activity slightly precedes the accumulation of droplets in the SPX, it may well be the source of this stainable material. Admittedly, the CHP and PAF techniques are selective rather than specific for neurosecretion (Bliss, Durand and Welsh, 1954), but previous work has shown physiological activity to be associated with the products of cells demonstrated with these processes (Passano, 1951a, 1951b, 1954). Carlisle (1954) has reported the movement of droplets from the ganglionic X-organs to the SPX. He suggests that the SPX is a point of storage and release for neurosecretory products. This is probably true of *Palaemonetes.* Since there was no accumulation of stainable material at the stubs of the brain in destalked larvae, the droplets found in the vacuole of the SPX probably were produced within the eyestalk. In adult eyestalks (Fig. 3), the axon of the giant cell can be seen to enter the neuropile of the *medulla terminalis* and is not directly incorporated into the sinus gland tract. The fibers could not be traced out of the neuropile.

Pyle (1943) described the position of the X-organ (SPX) in embryos of *Pinnotheres* and *Homarus*, as what will become the median ventral side of the eyestalk in the first zoea. This is very different from what I found in *Palaemonetes.* The SPX develops embryologically in that portion of the eyestalk that becomes the dorsal surface in the free-swimming larva. It remains dorsal throughout larval life, becoming ventrally located during the changes associated with metamorphosis. At metamorphosis, the eyestalk rotates on its longitudinal axis, the dorsal surface becomes ventral and the ventral, dorsal. All internal structures retain a constant relation to each other. It is interesting that shrimp zoeae swim upside down and backwards during larval life. At metamorphosis, the animal assumes the dorsal-side-up position of the adult. The sensory pore remains oriented toward the substrate throughout this change in attitude (Fig. 17). A functional difference between dorsal and ventral surfaces of the eyestalk was hinted at by Keeble and Gamble (1904), but their experiments involving changes in direction of illumination failed to support this idea. From the structural complexity of the SPX, especially in the adult shrimp, it is difficult to believe that the so-called sensory pore does not have functional importance.

The sinus gland cannot be seen in *Palaemonetes* until the fifth larval stage (Form V). Between this and final stage (Form VI), the nerve fiber endings comprising the sinus gland become increasingly compact. Staining reactions indicate that the structure does not become functional until post-larval life. Pyle (1943) found that the sinus gland was not recognizable until the third stage after hatching in *Homarus americanus.* By the fourth stage, the sinus gland had increased in size, but still failed to show the brilliant stain of the adult structure. Morphologically, the fourth stage in *Homarus* is a post-larva, in spite of its continued pelagic existence (Herrick, 1895). The activity of the sinus gland (*i.e.*, storage and release of neurosecretory products), seems to be limited therefore, to post-larval life. After metamorphosis, the internal development can no longer be related to external morphology. In *Palaemonetes*, the sinus gland appears to be functional after the second or third post-larval molt (Fig. 14). Because of difficulties with their rearing, Pyle was not able to study the development of *Pinnotheres* larvae. He does state, however, that the sinus gland was not evident in late embryos or in the first zoeal stage.

Broch (1960) reported that all larval stages of *P. vulgaris*, from hatching through the "8th zoeal stage," responded to background coloration with changes in chromatophores. He found that the chromatophores completely contracted over a white background and were fully expanded over a dark background. He stated that the red chromatophore, on an excised thoracic segment of a second zoea, contracted upon the addition of brain extract, expanded upon the addition of abdominal nerve cord extract, and contracted when eyestalk extract was added. It was not stated that the tissue extracts were of adult origin. Broch concluded (p. 306) that "endocrine control of chromatophores in the zoea of *P. vulgaris* is comparable to that of the adult."

Perkins (1928) showed that eyestalk removal resulted in dispersal of red chromatophores in adult *Palaemonetes*. Preliminary experiments with *P. pugio* larvae indicate that the response to eyestalk removal in larvae is opposite to that in the adult. Destalked larvae became pale following the operation; the pale condition persisted through metamorphosis. This reaction, opposite or otherwise, indicates that there is some influence exerted on the chromatophore system by larval eyestalks.

Costlow (1961) has reported finding a *Uca* black-dispersing substance in the eyestalk extracts obtained from zoeae of *Sesarma reticulatum*. The chromatophore-dispersing substance fluctuates in total activity during the molt cycle. Recognizing the possibility of different hormones controlling color change and molting, Costlow (p. 184) suggests the following hypotheses:

> "(1) The *Uca* black-dispersing substance located within the eyestalks of the larvae fluctuates because of changes in titre associated with moulting but is not directly responsible for, nor involved in, the actual moulting process, or (2) if the endocrine organs within the eyestalks of the larval stages are responsible for production or storage of several hormones, including the moult-inhibiting hormone, the reduction of the titre of the *Uca* black-dispersing substance may reflect the reduction in titre of the moult-inhibiting hormone also" (*sic*).

Results of the destalking experiment described above suggest that chromatophores and molting are under separate control in the larvae of *Palaemonetes*. Eyestalk removal definitely affected the chromatophores (contracted) of the larvae, while the operation seemed to have no influence on the frequency of molting.

The molt-inhibiting hormone has been related to the X-organ-sinus gland complex in all decapods studied (Carlisle and Knowles, 1959). In light of the historical evidence for lack of activity in the ganglionic X-organs, and the absence of a functional sinus gland during larval life, the failure of eyestalk loss to interfere with molting frequency does not seem unreasonable. If a molt-inhibiting hormone is present in larval shrimp, it is probably produced somewhere other than in the eyestalks. Possibly the molting cycle in the larva goes uninhibited. The rapid cycle (two-day intermolt) may go on continually as a function of the production of molting hormone by a larval molting gland, if one exists. The duration of proecdysis (Drach's stage D) in adult natantians represents 60+% of the molt cycle (Passano, 1960). Morphological details of new spine formation suggest that the proecdysal period may be almost continuous, or at least of longer relative duration, during larval development (Broad and Hubschman, 1963).

The accumulation of droplets in the larval SPX may represent the storage and/or release of a chromatophorotropic hormone. The deprivation of such a substance (or one component) results in the overall paling in larval shrimp. A similar material in the eyestalks of *Sesarma* larvae may be the *Uca* black-dispersing substance demonstrated by Costlow. It is noteworthy that *Sesarma* appears to be unique among crabs since it darkens in response to eyestalk removal (Enami, 1951; Prosser and Brown, 1961). This behavior is like that of adult *Palaemonetes* and opposite to the reaction in all other brachyurans. Carlisle (1955) has shown that molt inhibition and control of water balance are effected by separate hormones in *Carcinus*. If the relatively large size of destalked larvae is a result of increased water uptake, the control of these processes would seem to be separate in *Palaemonetes* larvae as well. The destalked larvae at metamorphosis were approximately 50% larger than the control animals. There was no difference in the duration of the intermolt periods between these groups. This suggests another possible factor attributable to the secretory products in the SPX.

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SUMMARY

1. The histogenesis of eyestalk organs in *Palaemonetes vulgaris, P. pugio, P. intermedius*, and *P. kadiakensis* is essentially the same. *Palaemonetes paludosus*, having an abbreviated larval development, follows the same sequence of events in a shorter period of time.

2. The developing eyestalk of *Palaemonetes* has two consistent, characteristic features: (a) a single, large, monopolar, secretory neuron that becomes active during the first larval stage, and (b) a sac-like vacuole in the sensory pore X-organ that appears to be the site of storage and release of secretory products, from the first larval stage through adult life.

3. The sensory pore X-organ develops from neuroblasts of the eyestalk ganglia. It appears 5 or 6 days before hatching. It is located dorsally and remains in this position throughout larval development. Secretory droplets appear in the SPX vacuole during the first larval stage. The accumulation of droplets increases throughout larval life.

4. The sinus gland is not recognizable until the fifth larval stage. It apparently does not function as a neurohemal organ until after metamorphosis.

5. At metamorphosis, the eyestalk rotates on its longitudinal axis. The dorsal surface becomes ventral and the ventral, dorsal. Since the animal turns over at this time, the orientation of the sensory pore remains constant with relation to the substrate.

6. Eyestalk removal had no effect on the larval molting cycle, nor on metamorphosis.

7. Eyestalk removal results in complete paling of larval *Palaemonetes.* The pale condition persists throughout larval development and metamorphosis.

8. There is no accumulation of secretory material in the brainstubs of destalked larvae. It is concluded that the droplets found in the SPX originate within the eyestalk.

9. It is suggested that the giant neuron is the source of stainable material found in the larval SPX.

10. The ganglionic X-organs are not functional during larval development.

11. The endocrine systems operating in *Palaemonetes* larvae differ in several respects from those of the adult.

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MARINE BIOLOG

SOME CYTOLOGICAL ASPECTS OF FERTILIZATION IN THE CROSS BETWEEN THE FUNA (CARASSIUS CARASSIUS, CYPRINIDAE) AND THE LOACH (MISGURNUS ANGUILLICAUDATUS, COBITIDAE)

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Because of their fundamental importance in biology, fertilization phenomena, particularly in crosses between different species, have attracted the attention of many zoologists, from both the cytological and the genetical standpoints. Pioneer contributions with fish in this field were made by Moenkhaus (1904), Morris (1914) and Pinney (1918, 1922, 1928).

Recently Suzuki (1953, 1955, 1957), dealing with embryological and morphological studies of artificial hybridization between the loach (*Misgurnus anguillicaudatus*) and the funa (*Carassius carassius*) or goldfish (*C. auratus*), reported that hybrid eggs showed high mortality at the gastrula and hatching stages of development. The larvae from the funa (♀) × loach (♂) cross survived for 24 days after hatching, while the larvae from loach × funa were extremely abnormal, showing edema. In development the larvae exhibited paternal influences in number of myotomes and melanophores and the size of the nuclei. The evidence presented seems to indicate that the fusion of the egg and the sperm nuclei might have occurred in fertilization. In artificial insemination with the sperm of the loach (*Barbatula toni oreas*) and the egg of the funa (*Carassius auratus*) made by Kobayasi and Yamabayashi (1958), a part of the eggs showed no observable abnormality in the course of their development; the larvae hatched and grew normally, with general external features characteristic of the funa. Quite recently, the present author (Kobayasi, unpublished) undertook a hybridization study between the funa and the loach (*Misgurnus anguillicaudatus*), identical with that of Suzuki (1953, 1955, 1957), and found that a part of the eggs from the cross developed normally, and that the larvae which hatched grew into fishes having the general features of the funa.

The greater part of the earlier work in this field has been morphological, and important cytological events regarding fertilization have remained unexplored. Cytological data are significant and interesting in providing a means of understanding the problems underlying fertilization phenomena. The present author undertook cytological investigations on the hybridization between funa and loach, with special attention to the behavior of spermatozoa during insemination and morphological features of the chromosomes in cleaving eggs.

MATERIALS AND METHODS

The hybrid combination dealt with in the present study was between the funa (*Carassius carassius*)♀, belonging to the Family Cyprinidae, and the loach

(*Misgurnus anguillicaudatus*)♂, a member of the Family Cobitidae. They were caught in a suburb of Asahigawa during the breeding season. Ripe eggs were obtained from funa females which had received injections of a suspension of loach pituitary bodies, and were placed in vessels containing water regulated at 27° C. Insemination was carried out artificially at temperatures from 26° C. to 28° C. The eggs derived from any single specimen were always divided into two lots, one of which was inseminated with the sperm of the loach, while the other lot was left without insemination for a control. In order to avoid any complications, insemination with the sperm of the same species was not performed in the present experiment. Certain numbers of eggs were taken out at required intervals for fixation. As a fixative, Bouin's solution was employed. Eggs obtained in July, 1960, provided the material for the present studies. Sections were prepared 10 micra thick by the ordinary paraffin method. They were stained with Delafield's hematoxylin and eosin. Smears of spermatozoa were made and stained with gentian violet.

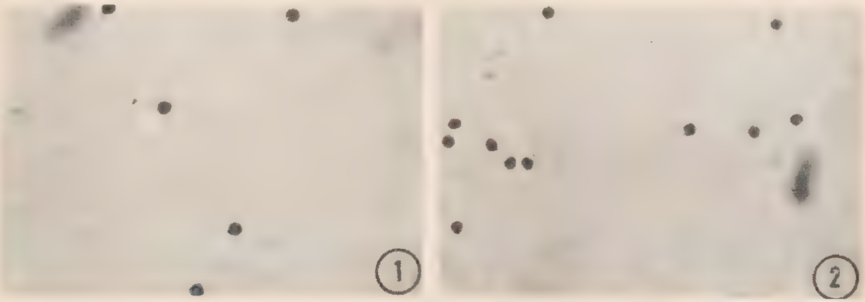


FIGURE 1. Spermatozoa of funa (*Carassius carassius*). × 600.
FIGURE 2. The same of the loach (*Misgurnus anguillicaudatus*). × 600.

RESULTS OF OBSERVATIONS

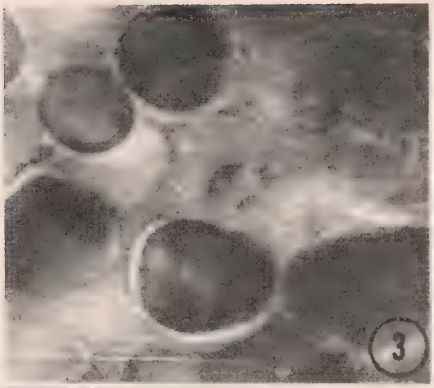
1. *Spermatozoa of the loach and funa*

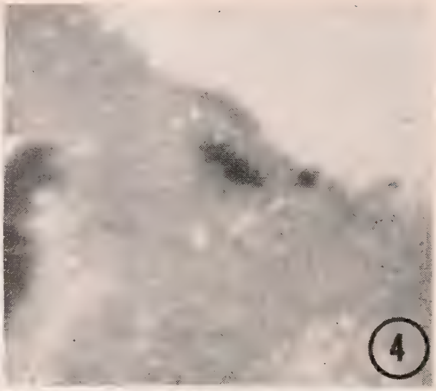
Spermatozoa of the loach and funa are similar, each consisting of a head, a middle piece, and a tail without any supplemental filament. The tail appears to attach to the margin of the middle piece.

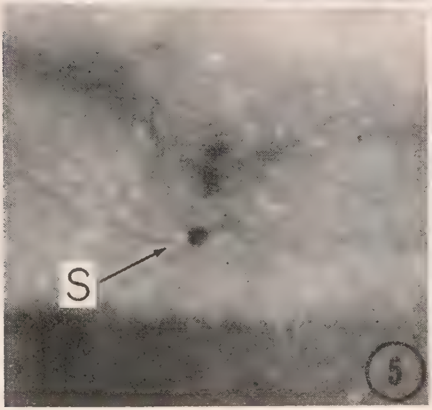
The spermatozoa of the two kinds of fishes differ from one another in form and size of the head, as well as in size of the tail. The head of the loach sperm is spheroidal, usually 3.0 micra in long axis and 2.8 micra in short axis, with a tail about 18.5 micra long. The head of the funa sperm is spherical, approximately 3.2 micra in diameter, having a tail about 14.5 micra in length (Figs. 1 and 2).

2. *The ovum of funa*

The ripe egg is nearly spherical in form, 1.4 to 1.7 mm. in diameter, with opaque orange coloration. The egg envelope is comparatively thick and tough, consisting of external and internal layers. The external layer is strongly adhesive when the egg is immersed in water. The micropyle, penetrating the enve-







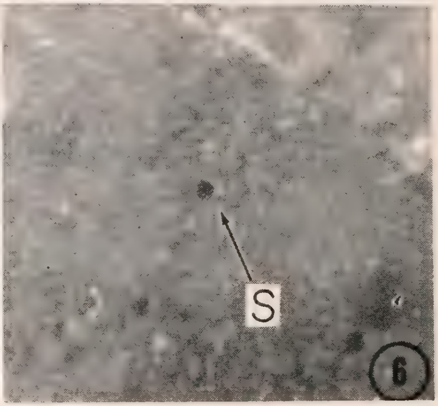


FIGURE 3. Second polar division, metaphase. From an egg of funa just after spawning, polar view. × 800.

FIGURE 4. The same view. × 800.

FIGURE 5. Insemination of loach sperm into funa egg through micropyle, just after insemination. × 800. S, sperm nucleus.

FIGURE 6. A sperm nucleus of the loach in funa egg, three minutes after insemination. × 800. S, sperm nucleus.

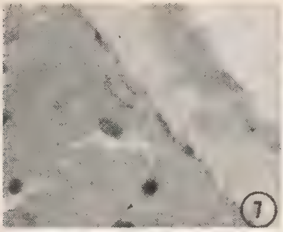
lope at the animal pole, is 5.5 to 6.2 micra in upper diameter, and 3.5 to 4 micra in lower diameter. The egg proper lies directly beneath the membrane. The cortical alveoli of various sizes are embedded in the cortical layer of the ooplasm. At the time of ovulation the egg shows the metaphase spindle of the second polar division lying in the periphery of the animal pole, nearly under the micropyle, in a slightly oblique direction (Figs. 3 and 4).

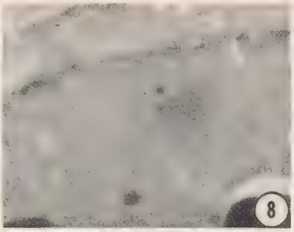
3. *The entry of the spermatozoon of the loach into the egg of funa and the formation of the pronuclei*

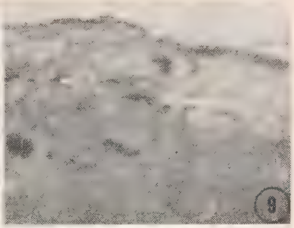
Sections of eggs fixed just after insemination showed the entrance of the spermatozoon into the egg through the micropyle (Fig. 5). In many eggs fixed 3 to 5 minutes after insemination, the penetration of the spermatozoon into the egg proper, together with the break-down of cortical alveoli, was observed (Fig. 6).

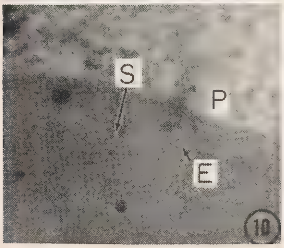
The sperm-tail could not be detected in the section. It was difficult to recognize clearly the middle piece in the fixed material. After insemination, the second polar division gradually progressed, with separation of the chromosomes at anaphase. With the passage of time, the chromosomes migrated to the opposite poles of the spindle (Fig. 7). The poly- or dispermic condition was not observed. Seven to 10 minutes after insemination, the sperm nucleus turned 180 degrees, and directed its base, with sperm asters, towards the center of the egg (Fig. 8). By this time the polar division was completed at telophase (Fig. 9). Then, the sperm nucleus developed into a vesicular body with the definite appearance of the astral rays, after which was formed the second polocyte. An egg taken at about 12 minutes after insemination, as seen in Figure 10, showed a vesicular sperm nucleus and the second polocyte. Generally, the second polocyte was completed about 15 minutes after the egg was inseminated.

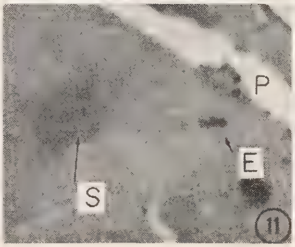
The chromosomes lying in the egg body became indefinite in outline and came together to form a mass of irregular outline (Fig. 11). In the meantime, a membrane appeared, surrounding the mass, although it is not well-defined. Then the nucleus became vesicular by vacuolization. In the meantime, the egg nucleus metamorphosed into a female pronucleus, inside which many weakly stained chromatin elements were scattered (Figs. 12–13). Along with the formation of the female pronucleus, the sperm nucleus completed its metamorphosis into a vesicular male pronucleus. It was nearly spherical in shape and smooth in out-











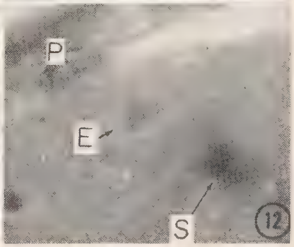


FIGURE 7. Second polar division, anaphase. From funa egg, 5 minutes after insemination. × 240.

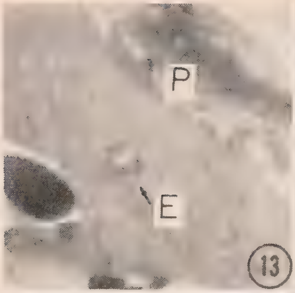
FIGURE 8. Sperm nucleus and sperm aster, 7 minutes after insemination. × 240.

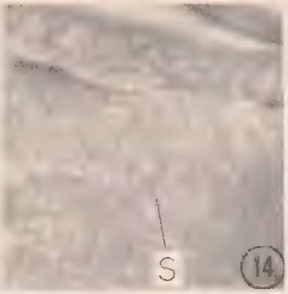
FIGURE 9. Second polar division, telophase, 7 minutes after insemination.

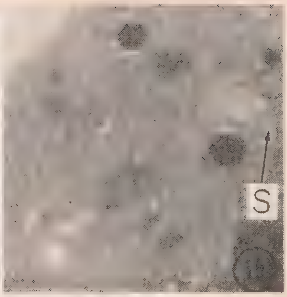
FIGURE 10. Extrusion of the second polocyte, sperm nucleus and sperm aster, 12 minutes after insemination. × 240. S, sperm nucleus. P, second polocyte. E, egg nucleus.

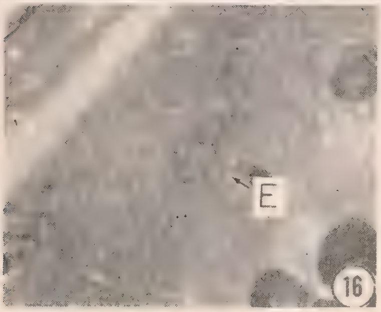
FIGURE 11. Second polar body, egg nucleus, and sperm nucleus before metamorphosis, 15 minutes after insemination. × 240. S, sperm nucleus. P, second polocyte. E, egg nucleus.

FIGURE 12. Second polocyte, egg nucleus before metamorphosis, and sperm nucleus in process of metamorphosis, 15 minutes after insemination. × 240. S, sperm nucleus. P, second polocyte. E, egg nucleus.









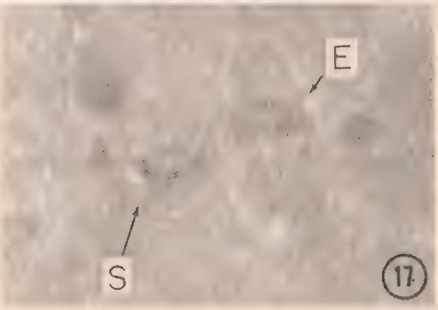


FIGURE 13. Female pronucleus in process of metamorphosis; from an egg, 15 minutes after insemination. × 300. P, second polocyte. E, female pronucleus.

FIGURE 14. Male pronucleus in process of metamorphosis, 15 minutes after insemination. × 300. S, male pronucleus.

FIGURE 15. Male pronucleus after completion, 20 minutes after insemination. × 300. S, male pronucleus.

FIGURE 16. Female pronucleus found in the same egg with Figure 15. × 300. E, female pronucleus.

FIGURE 17. Two pronuclei just prior to conjugation, 20 minutes after insemination. × 600. S, male pronucleus. E, female pronucleus.

line, carrying a fully developed sperm aster with radiation of rays (Fig. 14). It contained many minute chromatin bodies which stained weakly with hematoxylin.

At the time of completion, the female pronucleus lay in the egg periphery, being slightly larger in size than the male pronucleus. Figure 15 shows the latter at the stage of migration in an egg fixed 20 minutes after insemination. The female pronucleus found in the same egg is shown in Figure 16. Figure 17 is a section of an egg obtained 20 minutes after insemination, showing the male and female pronuclei just prior to conjugation. The astral radiation became inconspicuous with the migration of the two pronuclei to the deeper part of the egg.

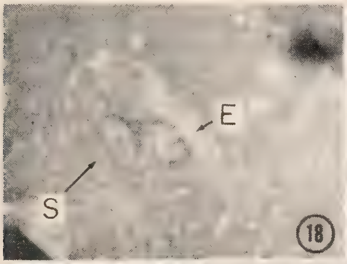
The course of metamorphosis of the sperm nucleus of the loach in the egg of the funa, as observed in the present study, seems to follow the pattern described by Ojima (1943) in the normally fertilized eggs of the carp, and by Yamamoto (1952) in those of the dog-salmon.

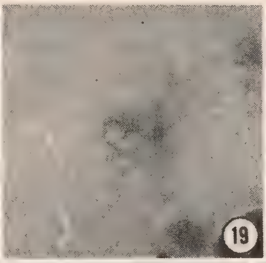
Unfertilized eggs of the funa were immersed in water and observed for control. They were activated in fresh water, and showed the breakdown of cortical alveoli, elevation of the chorion, and extrusion of the second polocyte. The time required for each stage in control eggs appeared to be nearly the same as that in experimental eggs. The unfertilized eggs remained without advancing further in development. A similar situation was reported by Yamamoto (1951) to occur in unfertilized eggs of the dog-salmon.

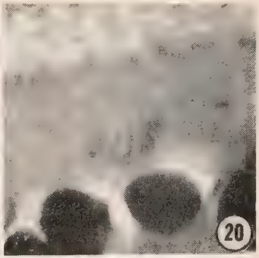
4. *Migration of the male and female pronuclei*

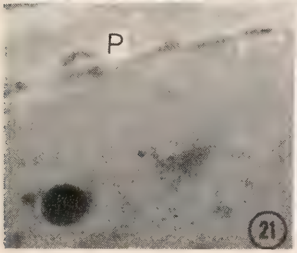
It is a matter of special note that the migration is more striking in the male pronucleus than in the female pronucleus. The penetration of the spermatozoon occurred always around the top of the animal pole where the micropyle exists. At first the spermatozoon was found lying in the periphery of the egg. About 15 minutes after insemination, or following the extrusion of the second polocyte, a migration of the sperm nucleus took place towards the deeper part of the egg, to conjugate with the egg nucleus.

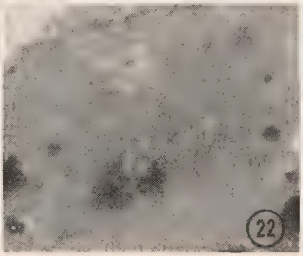
The second polar spindle was generally formed near the point of sperm penetration. After the extrusion of the second polocyte, the chromosomes left in the egg vacuolized into a vesicular body which was transferred into the female pronucleus. The latter moved slightly towards the center of the egg. About 20 or more minutes after insemination, the two pronuclei conjugated in the place where the female pronucleus already lay. After the conjugation the pronuclei persisted in their places throughout the whole period in preparation for the first cleavage. The pattern of the movement of the female pronucleus as observed in the present material is nearly similar to that reported in the carp eggs by Ojima (1943).











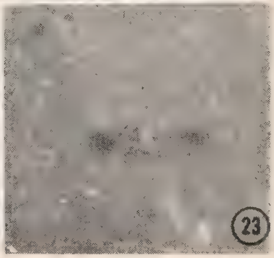


FIGURE 18. Two pronuclei during conjugation, 20 minutes after insemination. × 480. S, male pronucleus. E, female pronucleus.

FIGURES 19–20. Pronuclei after conjugation, 25 minutes after insemination. × 240.

FIGURES 21–22. Two centrosomes, and the first cleavage at prophase. From eggs about 25–30 minutes after insemination. × 240. P, second polocyte.

FIGURE 23. Prophase of the first cleavage. From an egg 30 minutes after insemination. × 240.

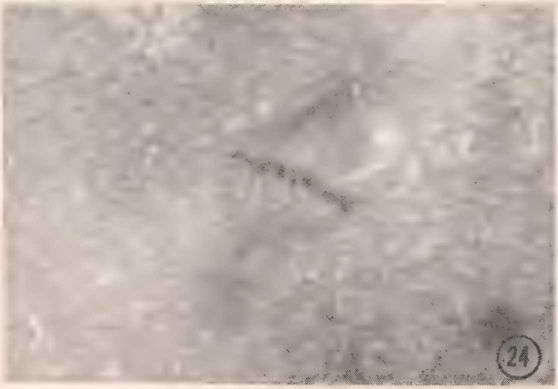
5. *Conjugation of the male and female pronuclei*

About 20 minutes after insemination, the male and female pronuclei conjugated in the eggs. The eggs obtained about 25 minutes after insemination showed in most cases the conjugation of the two pronuclei. In Figures 18 to 20, are shown the male and female pronuclei in process of conjugation.

In conjugation, they lie side by side with the nuclear membrane in intimate contact (Figs. 19–20). After conjugation, there is no actual fusion of the two pronuclei at all, but they keep themselves in close contact, distinctly separated by nuclear membranes. Further, this condition persisted unaltered throughout the whole period in preparation for the first cleavage. The staining capacity of the pronuclei increased gradually just after conjugation, but the male and female pronuclei were indistinguishable from each other by their staining capacity or size: they were quite alike in general appearance at the time of conjugation and there was no identifiable character by which they could be distinguished from each other. Sooner or later the astral system developed from the two centrospheres lying on opposite sides of the conjugation plane of the nuclei (Figs. 21–22). The astral system developed radial rays in every direction. At this stage, the chromatin elements in each nucleus became distinct and were well defined by their affinity for stain.

6. *The first cleavage*

Approximately 30 minutes after insemination had taken place, the great majority of the eggs examined were found at prophase or metaphase of the first cleavage division (Figs. 23–24). The eggs preserved about 35 minutes after insemination showed the anaphase or telophase spindle of the first cleavage. It was difficult to distinguish clearly the two different groups of chromosomes, paternal and maternal in their origin, at the stage of metaphase. At anaphase, each chromosome divided into equal halves and the halves migrated to opposite poles (Fig. 25). So far as the present observations went, the migration of daughter



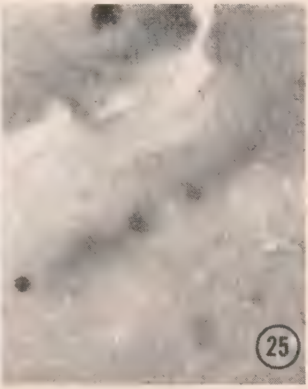
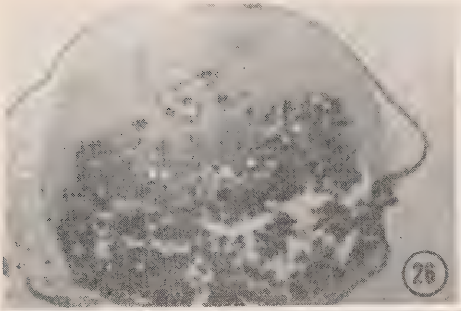
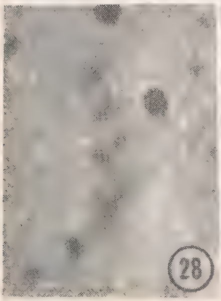
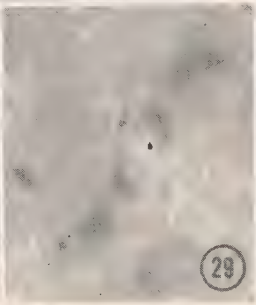


FIGURE 24. Metaphase of the first cleavage. From an egg 35 minutes after insemination. × 600.

FIGURE 25. Anaphase of the first cleavage, 35 minutes after insemination. × 300.







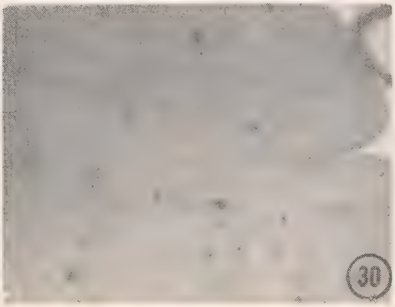


FIGURE 26. Two daughter nuclei after the first cleavage division. From an egg 40 minutes after insemination. × 150.

FIGURES 27–28. Anaphase spindle of the second cleavage. From an egg 60 minutes after insemination. × 300.

FIGURE 29. Anaphase spindles of the third cleavage. From an egg one and a half hours after insemination. × 75.

FIGURE 30. Metaphase of the sixth cleavage. From an egg two and a half hours after insemination. × 150.

chromosomes to the poles was almost synchronous, and abnormal behavior of chromosomes, such as elimination or lag of certain chromosomes, was not observed at all in the course of this division. The reconstruction of two daughter nuclei was completed about 40 minutes after insemination (Fig. 26). It is of interest that the second polocyte was found unaltered in its original position in the egg at the first cleavage.

It was by no means possible to count the actual number of chromosomes at metaphase or any other stages of the first cleavage.

7. *Early cleavages*

Early cleavages took place successively at intervals of about 15 to 20 minutes following the first cleavage. The resting stage in cleavage was short in time. The eggs taken about 50 minutes or more after insemination showed several blastomeres with spindles of various dividing phases. Though the observations were based on six eggs at early cleavage stages, the spindles of dividing blastomeres showed nothing irregular in the behavior of chromosomes; there was neither lag nor elimination of the chromosomes in the material so far observed by the author. Examples are shown in Figures 27 to 30.

Discussion

Moenkhaus (1904) made a cytological study of the process of fertilization in the cross between *Fundulus* and *Menidia*, and reported that about 50% of the eggs from the *Fundulus-Menidia* cross were dispermic, while in the reciprocal cross there were only a few which were poly- or dispermic. Loeb (1912), in discussion of the inheritance of omitted paternal characters in hybrids between *Fundulus* and *Menidia*, and between *Ctenolabrus* and *Stenotomus*, without cytological study of those eggs, stated that the sperm in these cases acted as a parthenogenetic agent. In the present study, artificial insemination of eggs of the funa (Cyprinidae) was undertaken with the sperm of the loach (Cobitidae). The results proved that monospermic insemination occurs in the normal manner, and that the head of the loach sperm metamorphosed into a male pronucleus, followed by conjugation with a female pronucleus of the funa egg. The cleavage of the hybrid eggs was found to proceed regularly. So far as the above features have shown, there is no evidence that, in the cross between funa and loach, the sperm acts as a parthenogenetic agent.

It was shown in the present experiments that after the penetration of the loach sperm into the funa egg, the metamorphosis of the spermatozoon and the egg nucleus into the male and female pronuclei, and the extrusion of the second polocyte in the egg, had taken place in a way comparable to that observed in the normal fertilization of some teleost fishes, for instance the carp (Makino and Ojima, 1943; Ojima, 1943) and the dog-salmon (Yamamoto, 1952). Also, the behavior of the pronuclei, before and after their conjugation in the egg, and the formation of the first cleavage spindle were proved to follow a normal pattern. After conjugation there was no actual fusion between the two pronuclei; a similar feature was reported in the fertilization of the carp (Ojima, 1943), dog-salmon (Yamamoto, 1952) and *Fundulus* × *Ctenolabrus* (Morris, 1914). This is a feature commonly found in other forms of vertebrates (*cf.* Wilson, 1928; Makino, 1934).

In eggs from the cross between *Fundulus* and *Ctenolabrus*, Morris (1914) observed that some chromosomes of male origin lagged at anaphase of the first and later cleavages. In the hybrid eggs from *Fundulus* × *Ctenolabrus*, *Stenotomus* × *Ctenolabrus*, *Fundulus* × *Prionotus* and *Menidia* × *Ctenolabrus*, Pinney (1918, 1922, 1928) announced that lag and elimination of some chromosomes of paternal origin occurred at anaphase of the first and later cleavages. So far as the present study is concerned, there is no evidence for the occurrence of abnormalities such as lag and elimination of the chromosomes. It should be mentioned, however, that the behavior of the chromosomes in the late stages of development remains unknown, due to poor material. Furthermore it was difficult to examine exactly the number of chromosomes in the cleavage spindle. Here, some note may be needed on the chromosomes of the loach and funa. It was reported by Makino (1939, 1941) that the diploid number of chromosomes was 52 in the loach (*Misgurnus anguillicaudatus*) and 94 in funa (*Carassius carassius*). Morphological difference between the chromosomes of the two species was very striking. In parallel to the systematically distant relationship between these two species, the chromosomes differed both numerically and morphologically. It is surprising to see that, between those two distantly related species, insemination and early cleavages took place without visible abnormality.

It was noted in the present material that the second polar body was found lying in its original position in the egg at the first cleavage. This is proof that the second polar body took no part in the formation of the first cleavage spindle.

Suzuki (1953) studied fertilization in the cross between the goldfish and loach and reached the conclusion that the hybrids might be produced as a result of the conjugation of the egg and sperm nuclei. But, there is no cytological proof for this conclusion. The present study seems to be of value in establishing with cytological evidence that, in the cross of funa × loach, insemination actually occurs and early cleavages of fertilized eggs proceed without detectable abnormality.

It is especially interesting to see that the cross between funa and loach yielded hybrid larvae growing in a normal manner (Kobayasi and Yamabayashi, 1958; Kobayasi, unpublished). In hybrid larvae derived from crosses with *Fundulus*, *Ctenolabrus* and some others, Morris (1914) and Pinney (1918, 1922, 1928) reported the appearance of abnormalities in greater or lesser degree. They showed further that there were lag and elimination of some chromosomes of paternal origin at anaphase of the cleavage of the hybrid eggs. The present author found that some hybrid larvae from the cross of funa and loach showed maternal characters very distinctly. However, since no abnormal division was observed in the cleavage of the hybrid eggs, the cause of the maternal effect remains in doubt and a subject for future investigation.

The author wishes to express his grateful thanks to Professor Sajiro Makino, Hokkaido University, for his expert guidance, helpful advice, and improvement of this manuscript. Cordial thanks are also offered to Mr. I. Yamabayashi for his kind technical aid in the course of this study.

SUMMARY

1. Insemination and early cleavages were cytologically investigated in the cross between the funa (*Carassius carassius*) and the loach (*Misgurnus anguillicaudatus*), belonging to different families. Eggs of the funa were artificially inseminated with loach sperm at water temperatures from 26° C. to 28° C.

2. Monospermic insemination was cytologically proved; a single spermatozoon of the loach entered the funa egg through the micropyle. Following insemination, the second maturation division of the egg proceeded. About 15 minutes after insemination, the extrusion of the second polocyte occurred. Then the formation of female and male pronuclei followed. About 20 to 25 minutes after insemination, the meeting of the male and female pronuclei took place. In conjugation, both pronuclei came into close contact, side by side, with the nuclear membrane intact, without evidence of actual fusion.

3. It was difficult to distinguish clearly groups of chromosomes of paternal and maternal origin or to count the chromosome number in the first cleavage metaphase. During the period from anaphase to telophase, separation of the chromosomes took place synchronously, without evidence of lag or elimination of chromosomes. The first cleavage was completed 35 to 40 minutes after insemination.

4. Early cleavages took place at intervals of 15 to 20 minutes after insemination. No abnormality was detected in the cleavage spindle. The second polar body was found lying in its original position at the first cleavage.

5. Evidence presented indicates that both insemination and early cleavage followed normal cytological patterns, and consequently that there is no cytological evidence in this cross to show that the sperm acted as a parthenogenetic agent, in spite of the maternal characters noted in larvae resulting from this cross.

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ACCUMULATION OF RADIOACTIVE CALCIUM IN LARVAE OF THE SEA URCHIN PSEUDOCENTROTUS DEPRESSUS

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The prominent role of calcium in the sea urchin egg, especially with reference to physiological events at the time of fertilization, has been repeatedly emphasized. But comparatively little is known of the accumulation of calcium and its utilization in the formation of the spicule during the course of development. Using radioactive calcium, Rudenberg (1953) reported that the *Arbacia* egg did not show any uptake of calcium up to the time of the swimming blastula stage, while the jelly coat accumulated calcium to a considerable extent. On the other hand, Hsiao and Boroughs (1958) reported that the unfertilized eggs of the Hawaiian sea urchin, *Tripneustes gratilla*, took up radioactive calcium even though the jelly coat was removed. From the observation that total calcium of the egg did not change after incubation with Ca^{45}, they concluded that Ca^{45} entered the egg cytoplasm by exchange with Ca^{40} of the egg. Örström and Örström (1942) found that the total calcium content of the *Paracentrotus* egg remained unchanged for a period of 15 hours after fertilization and then it quickly increased over 10-fold in the next 25 hours. They suggested that this increase was associated with the formation of the spicule. Recently, Yasumasu (1959) measured the calcium content of the spicule during development of the *Hemicentrotus* and *Anthocidaris* larvae and reached the same conclusion. Bevelander and Nakahara (1960) reported that in the *Echinarachnius* egg, the calcium accumulated in the areas of spicules and primary mesenchyme cells, and suggested that the calcium concentrated in the mesenchyme cells was transmitted to the growing spicules.

The present paper supplies some information concerning the uptake of radioactive calcium by sea urchin larvae at different stages of development, and the extent to which accumulated calcium is utilized in the formation of the skeleton. Preliminary experiments were carried out on *Paracentrotus* larvae by one of us (E. N.) at the Institute of Comparative Anatomy, University of Palermo, and the results obtained were briefly reported in Japanese (Nakano, 1960).

MATERIALS AND METHODS

The sea urchin used in the present experiments was *Pseudocentrotus depressus*, collected mainly at the Sugashima Marine Biological Station. The eggs were obtained by KCl-induced spawning and washed three times with filtered sea water. Dilute suspensions of eggs were fertilized and allowed to develop at room temperature (15–18° C.) under continuous gentle stirring.

In the first series of experiments, larvae were reared in the isotopic medium from the beginning of development at a level of radioactivity of 0.1 $\mu c./ml$. The concentration of added calcium (0.5 m*M*) was so low that no effect was found on the spicule formation, as reported by Okazaki (1956). At appropriate intervals, larvae were withdrawn from the culture and washed with sea water five times. They were then homogenized with 0.54 *M* KCl at pH 7.2 in a Teflon homogenizer of the Potter type. The homogenate (called "total homogenate") was centrifuged in the cold at 800 *g* for 10 minutes and the sediment was discarded, while the supernatant ("supernatant") was used for analysis. A 0.2-ml.-sample was placed on a stainless steel planchette, dried and counted with a thin end-window gas flow counter and an ordinary scaler. The samples for the determination of total nitrogen were combusted and the nitrogen was estimated by direct Nesslerization.

In the second series of experiments, larvae were reared in normal sea water until desired stages. They were then removed from the culture and incubated in the isotopic medium for two hours at room temperature. As the culture medium, artificial sea water containing Ca^{45} (1 μc./ml.) was used. After incubation, larvae were washed repeatedly with sea water and divided into two parts. One part was homogenized with 0.54 *M* KCl and used for the counting of radioactivity as described above, while the remaining part was used for the examination of autoradiographs *in toto* and in section. For making whole mount preparations, larvae were flattened and fixed with methanol between the slide and coverglass. After

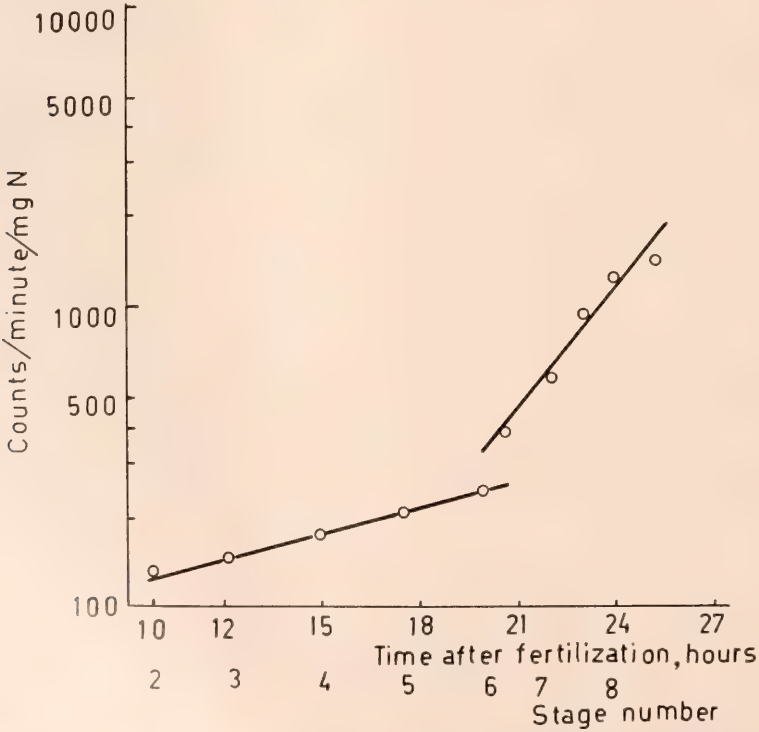

FIGURE 1. Total accumulation of Ca^{45} in *Pseudocentrotus* larvae during development. For the stage number see Table I.

TABLE I

Accumulation of Ca^{45} in Pseudocentrotus larvae during development. The values represent counts per minute per mg. nitrogen. Larvae were incubated at 18–19° C.

Stage number	Stage	Experiment 1		Experiment 2	
		Total	Supernatant	Total	Supernatant
1	4-cell	65	33	—	—
2	Blastula before hatching	133	131	—	—
3	Swimming blastula without mesenchyme cells	152	140	143	47
4	Early mesenchyme blastula	164	176	183	154
5	Mesenchyme blastula	205	195	241	177
6	Late mesenchyme blastula*	246	216	394	192
7	Early gastrula	596	201	987	257
8	Gastrula	1,290	208	1,985	305
9	Prism	15,530	1,082	11,280	1,036
10	Early pluteus	55,100	3,640	52,000	5,360
11	Pluteus	148,000	8,420	—	—

* Appearance of the spicular rudiment.

standing at least one hour, the coverglass was removed in water containing a trace of calcium (about 10 mM CaCl$_2$) and the slide was dried quickly after passage through graded ethanol. For making sections, larvae were fixed with methanol, and sections 10 microns thick were mounted on slides from which paraffin was removed by xylene. The slide was dried after passage through ethanol. All preparations were coated with a thin layer of celloidin without staining. Autoradiographs were made by the stripping emulsion technique, using Fuji autoradiographic plates. Exposure was completed in the light- and moisture-proof box at 2° C. for 2 to 10 weeks and developed photographically. Examination of the autoradiographs was made with both light and phase contrast microscopes.

RESULTS

When fertilized eggs were reared in sea water containing Ca^{45}, there was practically no accumulation of Ca^{45} during the early stages of development. At the early gastrula stage, however, the radioactivity of the total homogenate began to increase, showing the onset of calcium accumulation (Fig. 1). Thereafter, this accumulation increased rapidly and high radioactivity was observed at the pluteus stage (Table I). On the other hand, the radioactivity of the supernatant fraction remained relatively constant during gastrulation. Since the spicule is removed from the supernatant by centrifuging, the radioactivity found in the supernatant is not attributable to the spicule. It is highly probable that the supernatant contains bound calcium besides its free ions.

A rapid and abrupt accumulation of Ca^{45} in total homogenate at advanced stages of development suggests that the rate of calcium uptake may change during development. Figure 2 gives the time course of calcium uptake by *Pseudocentrotus* larvae at different stages. In these experiments, larvae were incubated in the iso-

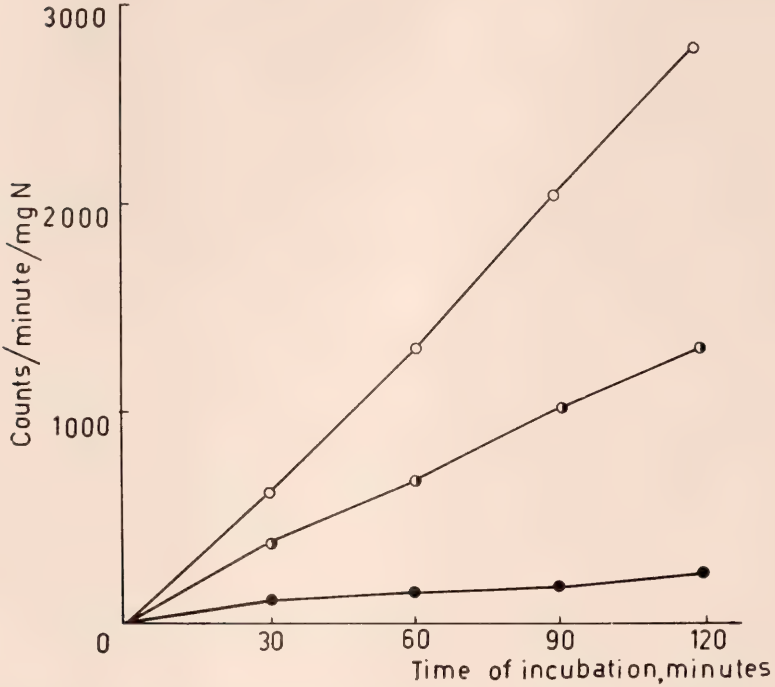

FIGURE 2. Time course of uptake of Ca^{45} at different stages of *Pseudocentrotus* larvae. Solid circles, blastula without mesenchyme cells; semi-solid circles, early gastrula; open circles, late gastrula.

topic medium at different intervals for a period of two hours and the radioactivity of total homogenate was counted. During gastrulation, the rate of calcium uptake increases rapidly and at the late gastrula stage, it becomes over 10 times greater than that of blastula without mesenchyme cells. These changes of calcium uptake were confirmed by autoradiographic techniques which ran in parallel with the above experiments. In the autoradiograph of the early gastrula, dark grains were found to be located in the two small areas, suggesting the onset of accumulation of Ca^{45} at the spicular rudiments (Figs. 3A and a). When the spicular rudiment develops into a triradiate spicule, its radioactivity increases markedly and this increase continues to the pluteus stage (Figs. 3B–E and b–e). Since these photographs clearly demonstrate that Ca^{45} is concentrated in the spicule, it seems likely that the rapid increase of calcium uptake observed by counting directly reflects the formation of the spicule.

Preliminary experiments on *Paracentrotus* larvae showed, however, that calcium uptake increases at the mesenchyme blastula stage before spicule formation. A detailed investigation of this point was carried out on *Pseudocentrotus* larvae. As shown in Figure 4, the rate of calcium uptake increases preceding appearance of the spicular rudiment. In accord with this finding, it was found in the autoradiograph of sections that dark grains were evenly distributed in the blastocoel before spicule formation (Fig. 5B). At the early stages of development, accumu-

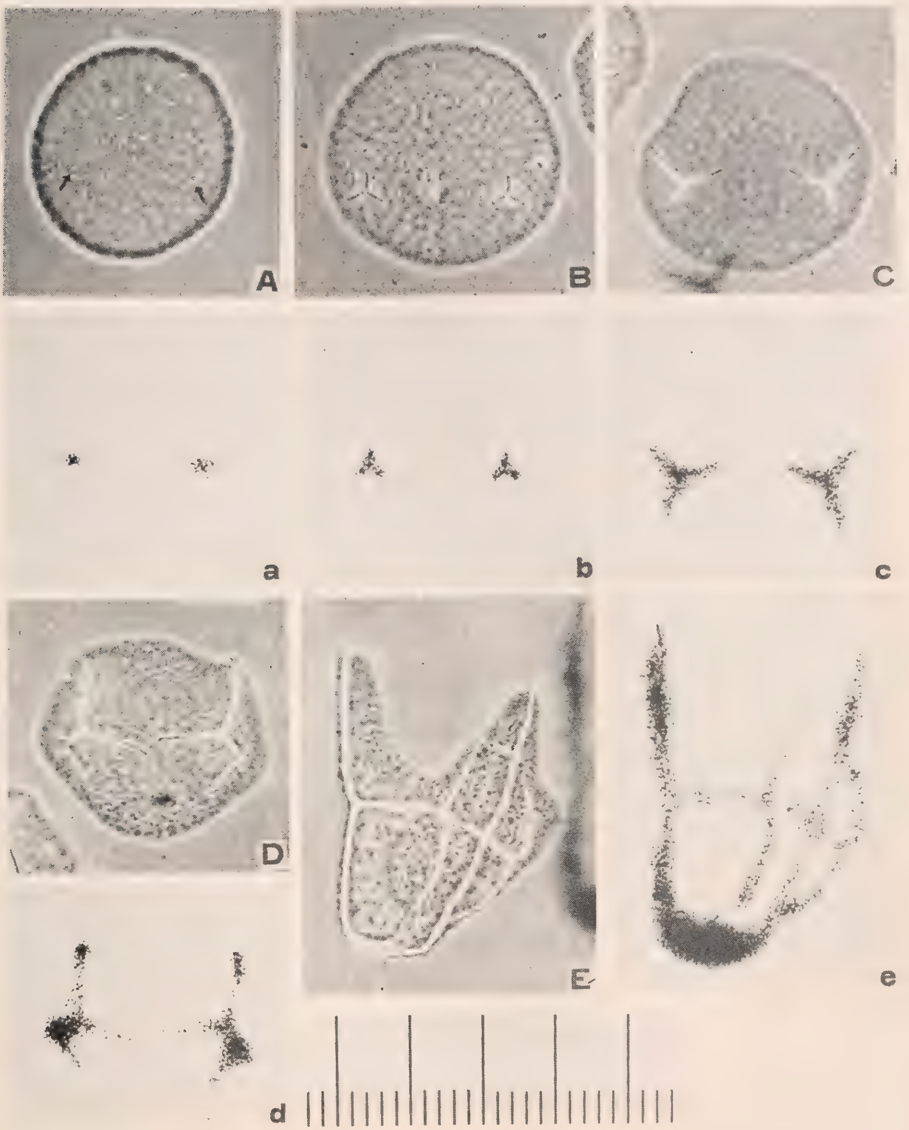

FIGURE 3. A–E, photographs of whole mount preparations of *Pseudocentrotus* larvae incubated in Ca^{45}-containing sea water for two hours, photographed by phase contrast optics. Arrows in A indicate the spicular rudiments. a–e, autoradiographs of the same specimens as in A–E, photographed by ordinary microscope. Autoradiographic emulsion was shifted after exposure to radiation so that dark grains were not superimposed over larvae.

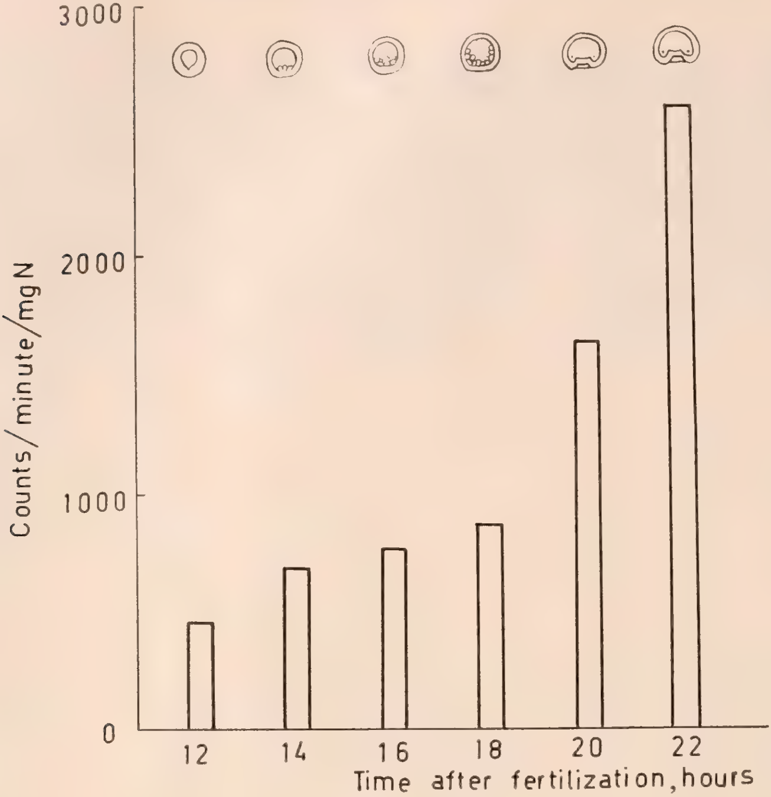

FIGURE 4. Rate of uptake of Ca^{45} by *Pseudocentrotus* larvae at different stages of development. Larvae were incubated in Ca^{45}-containing sea water for two hours. Upper diagrams show the stage at the end of incubation.

lation of Ca^{45} is insufficiently recognized in the blastocoel, as shown in Figure 5A. It may be assumed that permeability of larvae to calcium changes at the mesenchyme blastula stage and that the penetrated calcium is held as a Ca complex in the blastocoel before deposition on the spicule. There is no accumulation of Ca^{45} in mesenchyme cells.

DISCUSSION

The results of the present experiments show that the rate of uptake of Ca^{45} by sea urchin larvae is extremely low at the beginning of development. It rises at the mesenchyme blastula stage, which is followed by the second rise at the gastrula stage. Low uptake of Ca^{45} at the early stage may be attributable to an exchange of Ca^{45} with Ca^{40} of cytoplasm, as was pointed out by Hsiao and Boroughs (1958). Of the two steps of the increase, the latter one at the gastrula stage can be explained by deposition of Ca^{45} on the spicule, as assumed also from the data of previous workers (Örström and Örström, 1942; Yasumasu, 1959). The former increase at the mesenchyme blastula stage, however, seems to be additional infor-

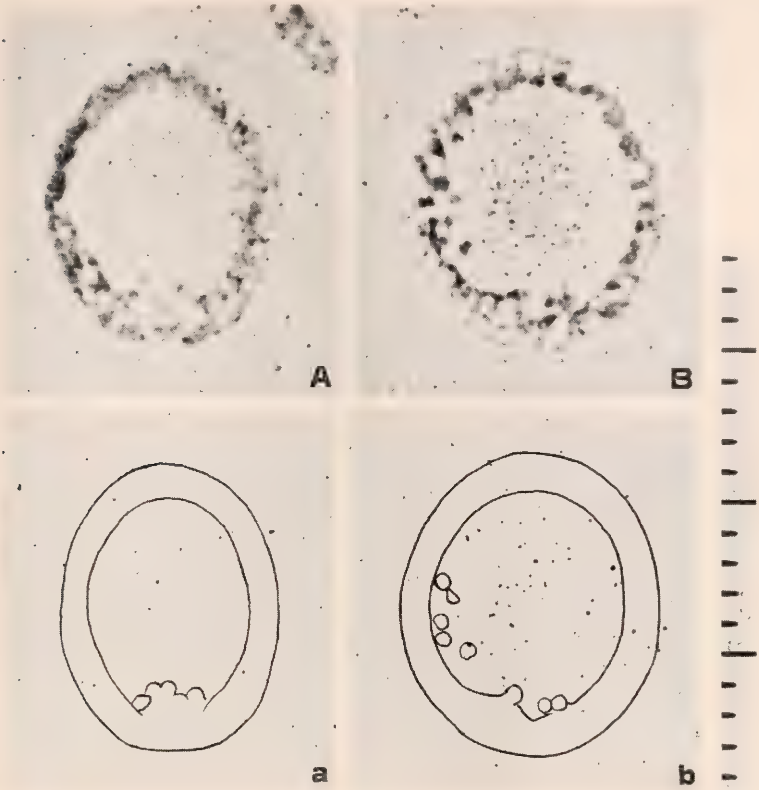

FIGURE 5. A and B, photographs of sections of *Pseudocentrotus* larvae incubated in Ca^{45}-containing sea water for two hours, photographed by phase contrast microscope. A, early mesenchyme blastula. B, late mesenchyme blastula before the formation of the spicular rudiment. a and b, autoradiographs of the same specimens as in A and B, photographed by ordinary microscope.

mation concerning the calcium metabolism in sea urchin larvae. By the autoradiographic analysis, Ca^{45} taken up by the mesenchyme blastula was found in the blastocoel. There is a possibility that Ca^{45} in the blastocoel may be bound with protein or mucopolysaccharide. In fact, blastocoelic gel was reported to be composed of mucopolysaccharides (Monné and Hårde, 1950; Monné and Slautterback, 1950, 1952; Immers, 1956, 1961). Bevelander and Nakahara (1960) supposed that calcium mobilized in the primary mesenchyme cells was transmitted to the growing spicules. But whether or not calcium is accumulated in these cells, as they claimed, is not clear from the present data. The investigation of this point will be a subject of future research.

From the fact that the rate of uptake of Ca^{45} begins to increase at the mesenchyme blastula stage, it seems likely that permeability of the larvae to calcium may change at this stage. In experiments with *Strongylocentrotus purpuratus*, Bolst and Whiteley (1957) reported that uptake of inorganic phosphate by the larvae increases rapidly at the early stage of development and then decreases with the onset of gastrulation. This pattern is entirely different from that of calcium uptake. It is of interest to note in this connection that the spicule of sea urchin larvae consists of calcium carbonate, not of calcium phosphate (Yasumasu, 1959).

SUMMARY

The uptake of radioactive calcium by sea urchin larvae was investigated. Up to the blastula stage, calcium uptake proceeds at an extremely low rate, while it increases greatly during gastrulation. Autoradiographs of whole larvae show the increase of calcium uptake in parallel with the formation of the spicule. Before appearance of the spicular rudiment, however, calcium uptake increases at the mesenchyme blastula stage. Autoradiographs of sections show that penetrated calcium is distributed in the blastocoel. It is assumed that calcium is concentrated in the blastocoel before deposition on the spicule.

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GROWTH EFFICIENCY IN ARTEMIA UNDER LABORATORY CONDITIONS

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Measurements of the efficiency with which animals can convert food into their own body tissue have been largely confined to the vertebrates of agricultural importance. Typical of the wealth of data in Brody (1945) are figures for milk production in cows of 33%, egg production in hens of 10%, and for Jersey cows from 0 to 2 years an efficiency decrease from 35% to 5%. Ricker (1946) quoted a decrease from 33% to 10% with increasing age for fish, and Ivlev (1939a) found a value of 31% for young carp. Within the invertebrates Ivlev (1939b) also recorded an efficiency of 32% for young *Tubifex*, Fox *et al.* (1948) 0.9% for the marine worm, *Thoracophelia mucronata*, and North (1954) 11% for *Littorina*. Richman (1958), using four different feeding levels on three different ages of *Daphnia*, found efficiencies in the adult from 17% to 10% for food levels of 25 to 100 cells/mm.^{3} (*Chlorella*), respectively. Gibor (1957), feeding young *Artemia* on *Dunaliella*, reported a 53% efficiency, and Lasker (1960), feeding the same genus to *Euphausia*, found a variation between 11% and 74%. Conover (1961) obtained values around 15% for *Calanus hyperboreus* IV and V feeding on *Thalassiosira*.

Little work so far has been undertaken on the many factors which might influence growth efficiency in an invertebrate. The work reported below is an attempt to monitor weight increase and food consumption of groups of animals, both throughout their life cycle, and in greater detail over a limited period of their growth.

MATERIALS AND METHODS

Dried eggs of *Artemia salina* (L.) were obtained from the Great Salt Lake, Utah. The alga, *Phaeodactylum tricornutum* Bohlin, originally sub-cultured from the Plymouth Marine Laboratory strain, had been maintained at Southampton for several years. The culture thrived in sea water, enriched only with inorganic nitrate and phosphate (Raymont and Adams, 1958). The sea water had a salinity of 35‰, and was filtered through two thicknesses of Whatman No. 1 paper and sterilized at 75° C.

The relationship between dry weight and length (from the anterior tip of the head to the base of the caudal furcae, following Gilchrist, 1959) was determined for *Artemia*, using nearly 80 separate samples at different ages. After being measured, the animals were rapidly washed in distilled water and oven-dried at 60° C. for three days. A torsion microbalance of the author's own design (Reeve, 1962) facilitated accurate weight determinations upon the smallest animals.

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In the first series of experiments, *Artemia* eggs were hatched and the nauplii kept without food for 36 hours while they used up their yolk reserves. Four batches of 100 nauplii (A, B, C and D) were then put in vessels in 50 ml. of culture medium at concentrations of 100, 75, 50 and 25 *Phaeodactylum* cells/mm.^{3}, respectively. A fifth vessel contained culture medium at a known concentration, but was without animals. This was to serve as a control to determine whether the plant cells were multiplying or sinking out of suspension. All five vessels were placed overnight in a dark cupboard at 20 ± 1° C. On the following day, a sample of liquid was withdrawn from each vessel, and its cell concentration counted with the aid of a Sedgewick-Rafter chamber. At the same time, all the animals were transferred from the vessels containing them to dishes, and all the vessels were re-filled with fresh medium at the original cell concentrations. The number of animals remaining alive was recorded and they were replaced in their respective vessels, which were then returned to the dark cupboard. This cycle of events was repeated daily.

The increase in feeding capacity of the animals, as measured by their depletion of the food cells each day, was such that the volume of their medium had to be progressively increased. By the time sexual maturity was reached, about five pairs of animals (male and female) remained in each experiment, and occupied 3000 ml. At this time there were from 30 to 60 survivors of the original 100, but it was necessary to reduce this number to avoid the handling of excessive volumes of sea water. The mature animals started mating and produced nauplii, which were counted and removed from the water daily. The experiments were brought to a conclusion after 55 days.

The cell counting technique consisted in taking ten random fields of the Sedgewick-Rafter chamber. The reliability of the mean of ten counts was estimated (Reeve, 1962) at ±10–15% at the 95% confidence level. Counts of the control suspension very rarely varied significantly in density over 24 hours.

The second series of experiments were undertaken to gather some information on the effects of the variation of temperature, salinity, and a greater range of food concentration on growth efficiency. It was decided, in order to restrict the work involved, to use animals over a limited period of their life cycle. Young animals, which had just used up their yolk reserves, were chosen. This ensured that all experiments began with animals in an equivalent physiological condition. The sex, phase of sexual cycle, and genetical differences between individuals, all exert progressively greater effects on feeding rates in *Artemia* as it gets older (Reeve, 1962).

Arbitrary standards of temperature, salinity, and food concentration were set at 20° C., 35‰, and 50 cells/mm.^{3}, respectively. Three groups of experiments were performed, in which one of these parameters was varied while the other two were held constant. Eggs were hatched in a medium of the same temperature and salinity as their respective experiments. The effect of temperature on growth efficiency in young *Artemia* was investigated using six temperatures ranging from 5° to 30° C. A series of salinities from 5‰ to 150‰ were employed in the salinity variation experiments. These were obtained by evaporating sea water at 75° C., and then diluting the concentrated brine as required. Six food cell concentrations from 5 to 200 cells/mm.^{3} were chosen to determine the effect of food concentration on growth efficiency.

TABLE I

Cumulative length, weight and number of offspring of animals; and number and weight of plant cells consumed and growth efficiency, in five-day intervals for experiments A–D. (Weight at birth has been taken into account in computing efficiency)

Exp.	Interval (days)	Length (mm.)	Number of young	Wt. animals + young (mg.)	No. cells eaten $\times 10^{-3}$	Wt. cells eaten (mg.)	Efficiency (%)
A	5	1.35		0.0040	613	0.0066	30
	10	3.30		0.0323	7,690	0.0830	39
	15	5.70		0.174	43,200	0.467	37
	20	8.50		0.575	121,000	1.31	44
	25	9.60	26	0.896	225,000	2.43	37
	30	10.60	113	1.40	347,000	3.75	37
	35	11.20	156	1.71	471,000	5.09	34
	40	11.75	198	2.01	588,000	6.35	32
	45	12.15	240	2.30	744,000	8.04	29
	50	12.35	339	2.63	932,000	10.1	26
	55	12.45	477	2.94	1,039,000	11.2	26
B	5	1.40		0.0039	589	0.0064	30
	10	3.60		0.0427	6,280	0.0678	60
	15	5.45		0.148	24,700	0.267	55
	20	8.10		0.501	76,900	0.831	60
	25	9.20	11	0.752	148,000	1.60	47
	30	9.90	62	1.07	236,000	2.55	42
	35	10.40	122	1.36	324,000	3.50	39
	40	10.80	146	1.55	409,000	4.42	35
	45	11.00	171	1.66	500,000	5.40	31
	50	11.10	223	1.83	596,000	6.44	28
	55	11.15	302	2.05	661,000	7.14	29
C	5	1.20		0.0032	401	0.0043	28
	10	2.25		0.0105	3,010	0.0325	26
	15	4.15		0.0661	13,300	0.144	45
	20	6.10		0.210	34,100	0.368	57
	25	8.50		0.575	74,000	0.799	72
	30	9.30	17	0.802	129,000	1.39	58
	35	9.80	28	0.939	174,000	1.88	50
	40	10.15	50	1.09	246,000	2.66	41
	45	10.40	91	1.27	338,000	3.65	35
	50	10.50	116	1.38	402,000	4.34	32
	55	10.55	182	1.56	517,000	5.58	28
D	5	1.00		0.0029	201	0.0022	41
	10	1.90		0.0063	916	0.0099	43
	15	3.00		0.0246	3,790	0.0410	55
	20	4.60		0.912	10,800	0.117	76
	25	6.15		0.219	25,500	0.275	79
	30	7.50		0.398	47,900	0.520	76
	35	8.30	9	0.546	75,200	0.812	67
	40	8.90	9	0.683	99,100	1.07	64
	45	9.15	35	0.811	133,000	1.44	56
	50	9.20	59	0.881	187,000	2.02	44
	55	9.20	100	0.984	224,000	2.42	41

The experiments began with 100 animals and proceeded as detailed for the first series, with regard to daily maintenance. They were concluded as the average length of the animals reached approximately 2 mm., which occurred within five days for those under the standard conditions. By the eleventh day, animals in all the experiments had attained this size, except those at 5° and 10° C. These two were growing so slowly that they were also discontinued on the eleventh day. Samples of about 15 animals were weighed at the end of each experiment, using the torsion microbalance. In this way, any weight increase could be directly determined, rather than having to rely on a length/weight relationship.

RESULTS

The efficiency of growth of *Artemia* $\left(\frac{\text{wt. of animal produced}}{\text{wt. of plant consumed}}\right)$ was computed for the first series of experiments in the following manner (see Table I). The 55-day period was divided into 11 successive 5-day units. Average values were extracted from the data for animal length, number of offspring, and number of *Phaeodactylum* cells consumed per animal to the end of each period. The first two quantities were transformed to estimations of dry weight, using the weight/length relationship. This was represented by a straight line (Fig. 1) over most of its length when plotted on double logarithmic coordinates. Cell numbers were related to dry weight, using the data of Raymont and Adams (1958), who found a million cells of this species weighed 0.0108 mg. The efficiency to the end of each period, or cumulative efficiency, in the four food concentrations could then be derived as in the last column of Table I. Figure 2 A–D (corresponding to experiments A–D) is a graphical representation, in which the growth rate curve for each experiment (as increase in length) has also been included.

It is clear that there is a definite peak efficiency which occurs about the time of inflexion of the growth rate curve. The peak is highest in the lowest food levels, although the rate of growth is slower, the onset of sexual maturity is later, and the broods and the ultimate size of the animals are smaller. Table I contains cumulative efficiencies up to the end of each successive five-day period. Efficiencies within each period, or moving efficiencies, may also be extracted by simple calculation, from this table. These indicate the actual efficiency level at which the food conversion process is working during the five-day period under consideration. A plot of moving efficiencies would follow those of cumulative efficiencies in Figure 2 fairly closely. This is because the progressively greater quantities involved, as the animals grow and eat more, tend to swamp the earlier smaller figures, and minimize the cumulative effect. One of the main discrepancies is to be found in the peak values, which, considered by themselves (without the cumulative effect of the previous values), would be up to 10% higher. The other is in the latter parts of the curves, which in Figure 2 are falling to values between 25% and 40%. The moving efficiencies would tend to level off to values of 15–25%. Stability is achieved because the animals have become relatively fixed in size, feeding rate, and reproductive capacity. This 15–25% could be called the "reproductive efficiency," or efficiency of production of offspring, and is highest in the lowest food concentrations, though the numbers of young produced are much lower.

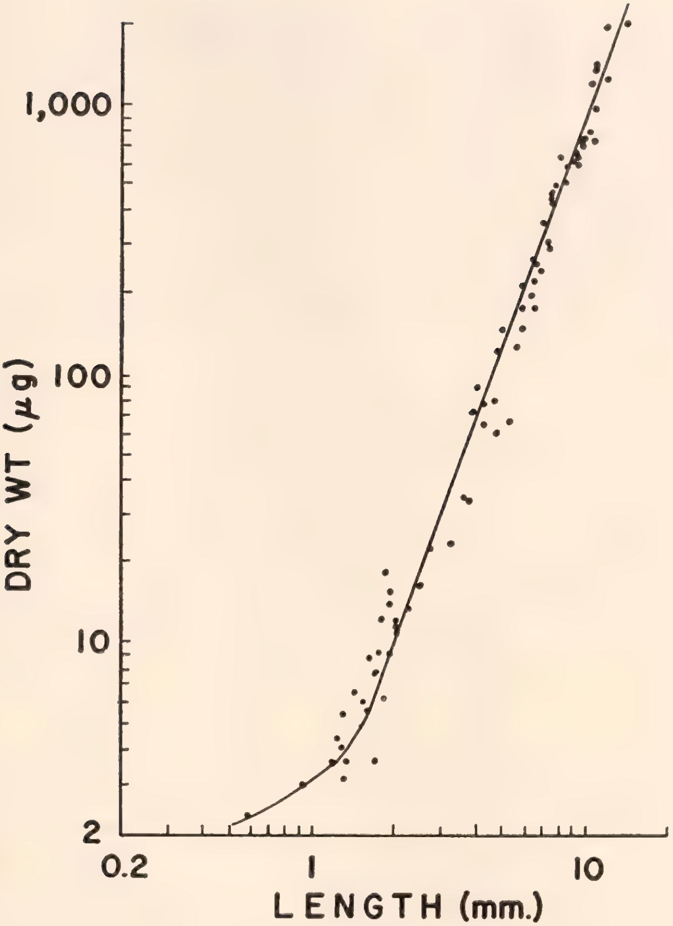


FIGURE 1. Weight/length relationship in *Artemia*.

These efficiency figures are average values. As the male animal reaches its maximum size and its rate of weight increase approaches zero, it continues to consume food. However, as this is used exclusively for non-tissue-forming metabolic functions, its growth efficiency also approaches zero. The female continues to use much of her food for the production of young, even after she has ceased to grow, and so maintains a high efficiency. Figure 2 assumes an "average" male/female animal, but in Figure 3 this average is separated into its male and female elements for experiment A. This is an alternative method of expressing the results, as weight of animal tissue produced against weight of plants consumed. Figure 3 is less sensitive than Figure 2 in demonstrating changes in efficiency during the earlier part of the life cycle of the animals. This is clear from the "average" curve for experiment D, which has also been inserted in Figure 3. This Figure is, however, useful in comparing the overall production of animal tissue in the two experiments. Although the food conversion process was more efficient in experiment D, the total production of tissue per animal was only 1 mg. compared with the 2.8 mg. of experiment A. The slope of the curves in Figure 3 at any given point represents the moving efficiency at that point. Reference slopes have been inserted around the margins of the figure.

Figure 4 summarizes the results of the second series of experiments, which was confined to young animals. All except two of the points on this figure indicate values for cumulative efficiency up to a length of 1.9 ± 0.1 mm. The exceptions were animals at 5° and 10° C. The former had lost weight slightly, and the latter had reached just over 1 mm. in length.

Figure 4A records the effect of a temperature increase, which resulted in a growth efficiency rise from zero at 5° C., where there was no weight gain, up to

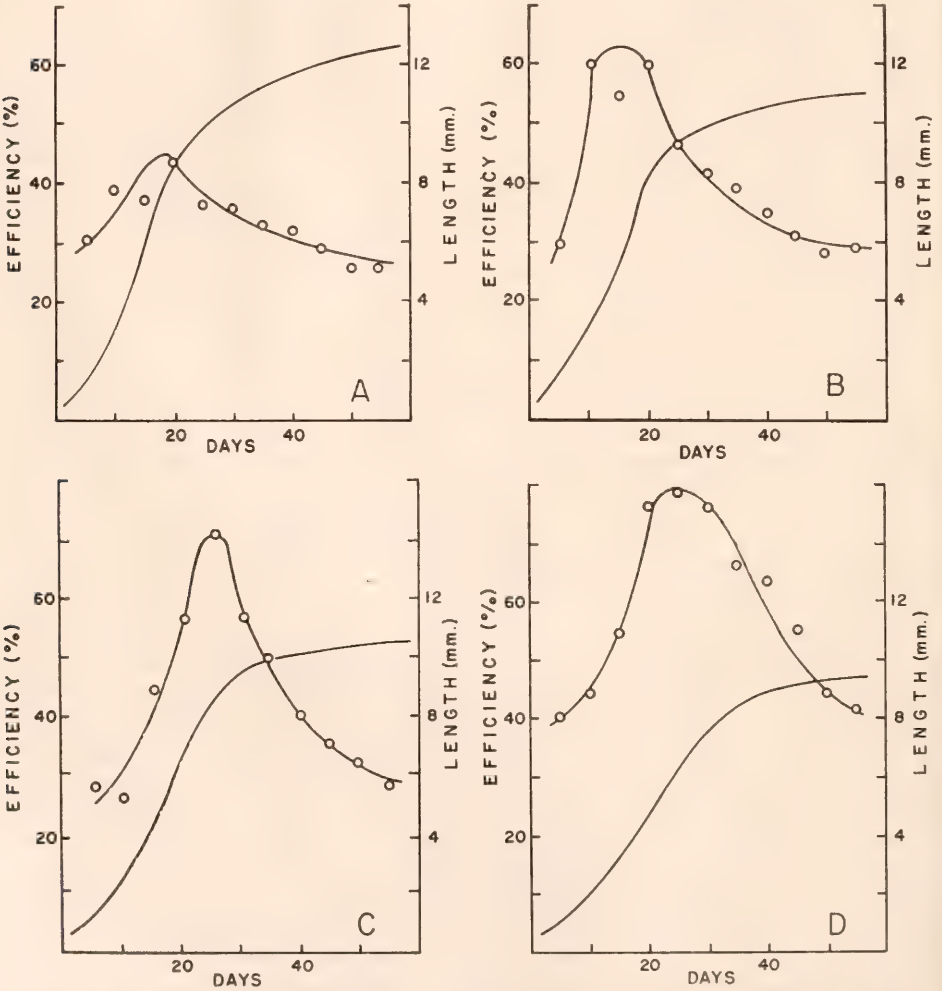


Figure 2. Cumulative efficiency (circles) and increase in length for experiments A–D.

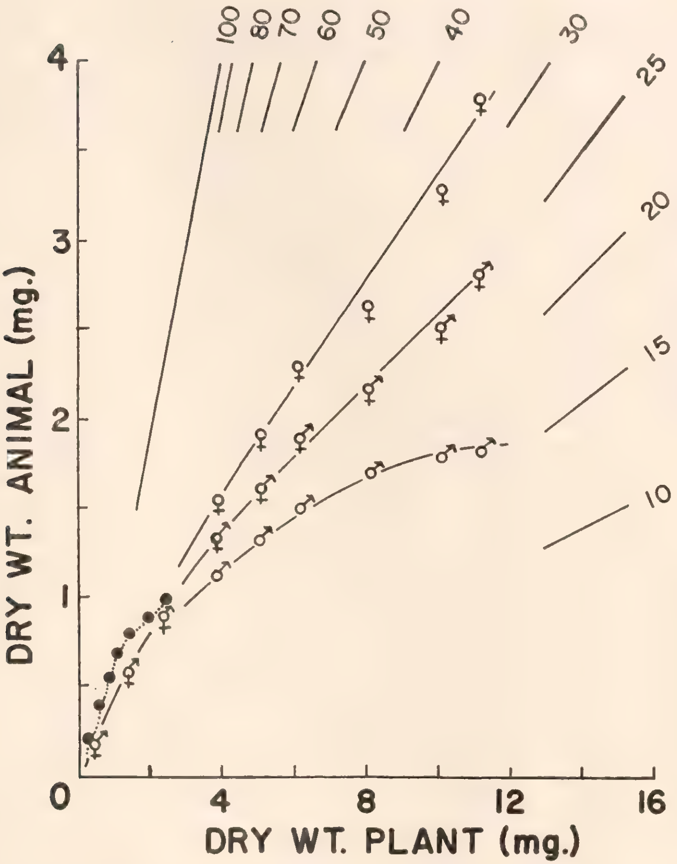
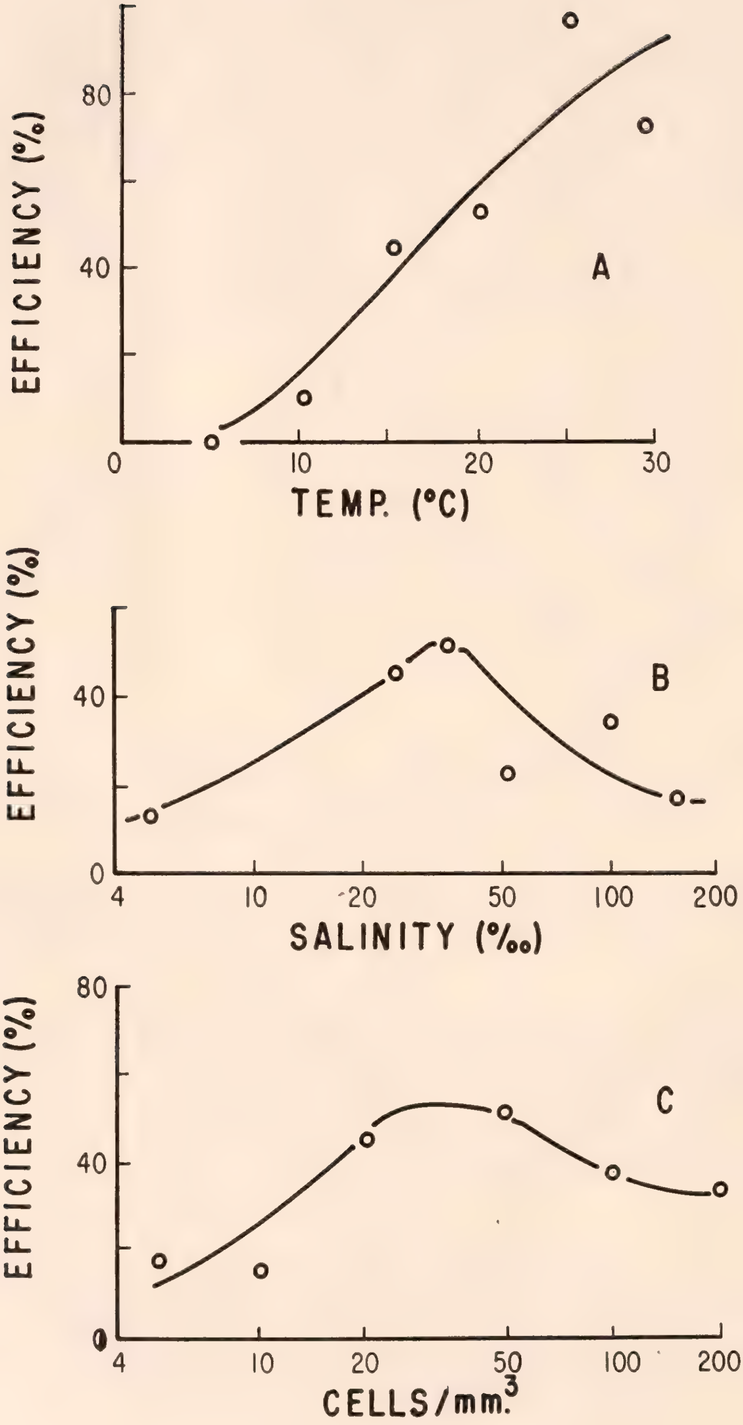


FIGURE 3. Gain in dry weight of animal tissue with dry weight of plant consumed for experiment A, with separate curves for male (♂), female (♀) and "average" (⚥) animal. Closed circles represent "average" animal of experiment D. Marginal slopes indicate percentage moving efficiencies.

about 85% at 30° C. Figure 4B, which plots the effect of increasing salinity, suggests a peak efficiency at 35‰ (that of normal sea water), which decreased as the medium became progressively more hypo- and hypertonic to this. As food cell concentration rose (Fig. 4C), growth efficiency increased at first rapidly, reached a peak and then fell away. The curve levelled off at the highest experimental cell concentrations.

DISCUSSION

The results presented above refer to an experimental single plant/animal system, and give a measure of "growth efficiency." The type of data derived from studies of natural communities has been differentiated by Slobodkin (1960) with the name "ecological efficiency." This author, and Richman (1958) may be consulted for reviews of synonymous and related terminology in the literature.


EFFICIENCY (%)
80
40
A
0
10
20
30
TEMP. (°C)
EFFICIENCY (%)
40
0
B
4
10
20
50
100
200
SALINITY (‰)
EFFICIENCY (%)
80
40
0
C
4
10
20
50
100
200
CELLS/mm.^{3}

Ecological efficiency, being related to an actual environment, is always the result of "averages, approximations, and generalizations" (Steemann Neilsen, 1961). In ecological surveys, values have been obtained ranging from 1% for conversion of carbon between primary producers and herbivores in continental shelf waters south of New York (Curl, 1961), to 75% for a temperate cold spring (Teal, 1957).

Growth efficiency is a figure which bears relation only to the physical and biological conditions of any particular laboratory experiment, and cannot be directly related to figures derived from ever-changing natural habitats. Strict comparisons cannot even be made between growth efficiency figures from different workers, where units range from dry weight (Ivlev, Ricker) through calorific value (Richman) to radio-active carbon (Lasker). Despite these reservations, the work of previous authors may be contrasted with the results presented above for *Artemia*, in which growth efficiency increases at first during the early life of the animal. Observations on the invertebrates are scant and mostly single values (Gibor), or for a single age (Lasker). Richman dealt with only three points in the life cycle of *Daphnia*, and found that efficiency decreased with age. It is possible that an efficiency peak could have occurred between the first two points.

Brody (1945) did not preclude the possibility of such a peak when he stated (p. 49), "the increase in size of a given animal associated with increasing age would be expected to increase the energy cost of its maintenance, and reduce correspondingly the total efficiency of growth unless this increase in maintenance is compensated by an increase in growth rate." It appears that in *Artemia* the growth rate is such that the increase in maintenance is more than compensated, since it has been shown (Fig. 2) that growth efficiency not only fails to decrease during the early life of the animal but shows a marked upward trend.

The higher peak efficiencies in the lower food levels may be correlated with the observation (Reeve, 1962) that food remains in the gut progressively longer, as there is less pressure on it from the incoming food to move through the gut. Presumably, the longer stay in the gut permits greater digestive efficiency. A similar inverse relationship was found by Richman (1958). These high peak values may be compared with 73% for *Mytilus* veligers (Jørgensen, 1952), 16–74% for *Euphausia* (Lasker, 1960) and figures for pre-natal growth of embryos of hen, frog, fish and silkworm of 52%, 51%, 59% and 65%, respectively (Brody, 1945). These figures imply very high digestive efficiencies within the range that have been reported by Marshall and Orr (1955) for *Calanus*, Lasker (1960) for *Euphausia* and Berner (1962) for *Temora*. All three workers found values of over 90%. If *Artemia* is capable of assimilating such high percentages of its ingested food, then peak growth efficiencies of up to 80% are theoretically possible.

In order to measure directly the weight of animals as they grow, a sample of the population must be sacrificed at frequent intervals. To do this the population must be either inconveniently large, or the work must be restricted to a small part of the life-cycle. The former situation was avoided by using length as an index of weight in the first series of experiments; the second series was of short duration, and the animals were directly weighed at the conclusion of each experiment. The results of the second series may thus have been determined with greater accuracy,

FIGURE 4. Relationship between cumulative efficiency and (A) temperature, (B) salinity, and (C) food concentration in young animals.

since the intermediate stage of computation was eliminated. As before, efficiencies were computed in general over no less than a five-day period. It must be noted, however, that the figures so derived did not necessarily represent animals of equivalent physiological ages. Discrepancies were most marked in the lower temperature experiments, where animals did not grow enough to reach the length of 2 mm., arbitrarily set for the conclusion of each experiment. At 5° C., only one-third of the animals had passed through their first molt, and averaged no more than 0.5 mm. in length by the eleventh day.

Efficiency in young animals varies markedly with salinity, and there appears to be an optimum corresponding to normal sea water. This is further evidence that the brine shrimp is not physiologically adapted to high salinities, any more than it is by virtue of its ionic regulation mechanism (Croghan, 1958), which has such a wide tolerance range that the animal can exist in water down to 15% normal sea water. Though Potts (1954) computed that the proportion of the total work expended on maintaining osmotic equilibrium in reduced salinities is theoretically very small, Croghan (1961) pointed out that such minimal levels are not likely to occur in practice. The osmotic work involved in keeping an internal fluid hypotonic in strong brines is not known. It may be that the decrease in growth efficiency, in both hypo- and hypertonic sea water, is at least partly due to increased proportions of assimilated food being appropriated for osmotic work. Beament (1961) developed the hypothesis that physiological specialization is far less significant than behavioral specialization in restraining animals to particular environments. As others have suggested, it may be the inability of *Artemia* to defend itself from predation which is important in limiting its natural habitat to saline lakes.

Although *Artemia* has been shown to utilize its food more efficiently in lower cell concentrations, this trend must ultimately reverse and drop to zero. This would occur at the cell concentration when the food passing through the gut is sufficient to do no more than provide for maintenance, leaving nothing available for building new tissue. In young animals (Fig. 4C) this trend is evident, with a maximum growth efficiency occurring in a food level between 25 and 35 cells/mm.^{3}. At higher cell concentrations, efficiency falls as the food moves through the gut faster, until about 200 cells/mm.^{3} when it levels off. This cell concentration has been correlated in young animals (Reeve, 1963) with that at which a maximum rate of ingestion is reached, *i.e.,* in cell concentrations above this, food moves through the gut at the same constant rate.

In this work, precautions were taken to reduce any effect due to bacteria, by using sterilized water in both plant and animal cultures. The experimental media were changed daily, and the plant cultures were rejected at any sign of a bacterial buildup, and in any case were used only in their rapidly growing phase. However, the experiments were undoubtedly not completely bacteria-free, and hence all efficiencies will have been slightly overestimated. Antibiotics were not used to kill bacteria, as they were suspected to have a depressive influence on filtration rate in copepods (Conover *et al.,* 1959).

These feeding experiments serve to indicate several factors which can influence growth efficiency, even though they may be claimed only as preliminary in the field of continuous measurement throughout life of an invertebrate laboratory population. The combined effect of all the errors involved in these determinations cannot be estimated with any accuracy. The insensitive logarithmic length/weight relationship used in the first series of experiments, and the small size of the young animals, would probably combine to render the earlier parts of the efficiency curves least accurate. For instance, the animals of experiment C, unlike the others, appear to have attained only 30% efficiency after 10 days of growth, although from Figure 4C of the second series, a value of about 50% was recorded in a comparable experiment. This is, in all probability, an extreme case. However, there is little doubt that efficiency peaks exist, and are related to food concentration and growth rate.

There have been several attempts to raise the productivity of natural aquatic environments by adding inorganic fertilizers, in order to obtain an eventual increase in the yield of animal tissue (*e.g.,* Gross *et al.,* 1950). By dependence on a transfer of energy through several links of the food chain, the results of these attempts have proved uneconomical and difficult to predict. Direct harvesting of mass cultures of algal cells (Burlew, 1953; Raymont and Adams, 1958) is also prohibitively expensive. Raymont (personal communication) suggested that experiments intermediate between these two extremes should be undertaken. A filter-feeding herbivore might be used to harvest the algal cells, because the animal would be relatively easy to remove from the environment. The work reported above indicates that the conversion of plant to animal tissue, could, by careful choice of conditions, be maintained at an efficiency well over 50%.

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ADDENDUM

Since the preparation of this paper, Mason (1963) reported some feeding experiments with *Artemia,* which may be compared with those of this work. In this work the volume of the medium was adjusted often as the animals grew. This was to ensure, as far as possible, that the cell depletion over 24 hours exceeded that required for statistical significance, but was not more than 50% of the original concentration. Mason was concerned mainly with offering a constant specified number of food cells to his animals each day as they grew. Although some of his experiments were begun daily at a variety of food concentrations, he reported that the nauplii consumed up to 99% of the food in 24 hours. In the first series of experiments the growth of his animals was severely stunted by the lack of an adequate supply of food. Under such conditions no conclusions may be drawn concerning the effect of cell concentration upon growth rate. Under natural conditions it is generally the concentration, rather than the absolute amount of food in the environment, which is of importance to the filter-feeder from day to day. The very low growth efficiencies recorded by Mason may be partly a reflection of the fact that his animals were progressively more limited by their food supply. It may be pointed out that he finds higher efficiency values during the active growth phase of

Artemia. In short-term experiments with adult animals he obtained an average of 17% for growth efficiency. This is much closer to figures reported in the present work.

SUMMARY

1. The efficiency of growth of four laboratory populations of *Artemia* in different food concentrations was studied over a period from birth to approximately double the time required to reach sexual maturity. Uptake of food and increase in length, and hence weight, were measured daily. Efficiency rose at first, up to the time at which the animals were growing at their maximum rate, when the highest peak cumulative efficiencies (79%) were obtained in the lowest food concentrations. These values then fell as the animals approached their maximum size.

2. In other experiments on young animals in their first few days of growth, it was found that efficiency increased with temperature between 5° and 30° C., that there was an optimum salinity of about 35‰, at which efficiency was greatest, and an optimum food concentration of 25–30 cells/mm.^{3}.

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REPRODUCTION OF THE BAY SCALLOP, AEQUIPECTEN IRRADIANS LAMARCK. INFLUENCE OF TEMPERATURE ON MATURATION AND SPAWNING[1]

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Influence of temperature on reproduction of marine invertebrates has been extensively reported in the literature. In many of these studies, reproductive cycles and spawning periods were described for a population of a species in one geographical area. Orton (1920) considered temperature as the most important factor for regulation of breeding in marine animals. Although he indicated that under normal conditions breeding temperature for a species is a physiological constant throughout the range, subsequent workers have demonstrated that latitudinally separated populations of a species breed at different temperatures (Loosanoff, 1956; Korringa, 1957). Hutchins (1947) stated that critical sea temperature for both reproduction and completion of the life cycle plays an important role in defining the distributional range of a species.

The differences in reproduction and larval ecology of widely distributed marine invertebrates are discussed in detail by Thorson (1950). The variation in reproductive physiology at different latitudes of some marine invertebrate species has been discussed in a recent review by Giese (1959). Loosanoff (1956) studied the temperature requirements for maturation and spawning for transplanted southern populations of *Crassosstrea virginica* in northern waters. Experimentally, the gonads of *C. virginica* and *V. mercenaria* were ripened in winter and the larvae were reared in the laboratory (Loosanoff and Davis, 1950, 1952). Turner and Hanks (1960) reported stimulation of gametogenesis in *Hydroides dianthus* and the bay scallop, *Pecten irradians,* during winter at higher temperature in the laboratory.

Aequipecten irradians Lamarck has a wide geographic distribution along the Atlantic and Gulf coasts of the United States, resulting in widely separated populations being exposed to different temperature regimes. In addition, morphological differences between populations have been reported and it is possible that physiological differences also exist (Abbott, 1954; Sastry, 1961). The present investigation was undertaken to study the influence of temperature on maturation of gametes and spawning of a population of bay scallops from Florida. These results were compared with data obtained on more northern populations by Belding (1910), Gutsell (1930) and Marshall (1960)

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MATERIALS AND METHODS

The animals were obtained from grass flats in Alligator Harbor, Franklin County, Florida. Determination of the natural reproductive cycle was made on samples of animals collected at monthly intervals. The gonadal condition was determined by microscopic examination and gross observations of the coloration of the gonad. On the basis of these two criteria, the functionally bisexual gonad was classified into one of six stages of development. These stages are described in detail below. Animals obtained from their natural environment were also maintained in the laboratory in running sea water at 23.0 ± 1.0° C. for the purpose of studying the influence of temperature on gonadal maturity and spawning.

Spawning was induced in mature animals in the laboratory, by heating. Mature scallops were placed in fingerbowls containing sea water of the same temperature as that at which they had been maintained. The temperature of the sea water was gradually raised to 30° C., by heating with a 115-watt lamp, and allowed to cool gradually. The temperature of the sea water in the bowl at the time the animal spawned was recorded and is considered as the spawning temperature. The procedure for stimulation of spawning in the laboratory was as shown in Table I, to study the effect of changing temperature and light on gamete liberation.

TABLE I

Method of changing temperature and light to stimulate spawning in the bay scallops in the laboratory

Bowl	Temperature °C.	Light
1	Initial increase and later cooling	exposed
2	Increase only	exposed
3	No increase or decrease	exposed
4	Initial increase and later cooling	not exposed
5	Increase only	not exposed
6	No increase or decrease	not exposed

OBSERVATIONS AND RESULTS

Morphological differences in geographic populations

Aequipecten irradians Lamarck is a polytypic species according to the classification of Dodge (1952) and Abbott (1954). Three subspecies, *A. irradians irradians* Lamarck, *A. irradians concentricus* Say and *A. irradians amplicostatus* Dall are recognized, based on the ecology and morphological characters of different geographic populations.

Rib counts on the outer side of the left valve were made for the Alligator Harbor population and compared with those of specimens obtained from Beaufort, North Carolina, and Woods Hole, Massachusetts. While the number of ribs in the Alligator Harbor population varied between 18–24, 21 being the most common, Beaufort and Woods Hole scallops had 18–19 and 17–18, respectively.

The temperature zonation on the Atlantic coast of the United States has been shown by Fischer (1960), indicating the differences in summer and winter temperature ranges above and below Cape Hatteras. Scallops in the Woods Hole region are subjected to cold temperatures in winter whereas the Beaufort and Alligator Harbor scallops experience much warmer temperatures throughout the year.

Reproductive cycle

The gonad condition of bay scallops obtained from Alligator Harbor is classified into stages I–VI. Stages I–III are immature, IV is mature, and V–VI are partially spent and spent conditions. Stage I gonad is small and transparent, and the only reproductive tissues that were observed were narrow tubules with primary germ cells. The stage II gonad has increased in size and is translucent. In gross examination, testicular and ovarian regions could not be distinguished; however, microscopic examination revealed that a few follicles had developed spermatogonia and oogonia. The gonad in stage III began to fill in with spermatogonia and oogonia and it became enlarged in volume. The bisexual nature of the gonad could be seen from the proximal white testicular area and the distal pale orange ovarian portion. Spermatogonia increased in number and were in clumps. A few spermatozoa were also present. Many half-grown oocytes with stalks and a large germinal vesicle were observed.

TABLE II

Gonad condition of bay scallops during 1957–1958

Month	Number examined	Condition of gonads per cent		
		Immature stages I, II, III	Mature IV	Spent V, VI
November, 1957	5	100	0	0
December	—	—	—	—
January, 1958	36	100	0	0
February	133	100	0	0
May	4	100	0	0
June	148	94.6	5.4	0
July	166	53.6	46.4	0
August	136	0	61.0	39.0
September	35	0	53.6	45.7
October	21	0	100.0	0
November	16	0	93.7	6.3
December	7	0	86.4	13.6

The stage IV gonad has increased considerably in volume, with round margins; it contained thickly packed follicles. The testicular and ovarian portions were cream and bright orange, respectively. Microscopic examination showed free, active spermatozoa and mature pear-shaped oocytes. In the partially spent gonad condition (stage V), testis and ovary were differentiated by the pale white and orange colors of their respective regions. The gonads retained some residual mature genital products. Empty spaces in the follicles of stage V gonads distinguish them from stage III gonads. Completely spent stage VI gonads were light brown in

TABLE III

Stimulation of the bay scallops to maturity in running sea water in the laboratory

Date of collection	Number of animals used	Condition of the gonad at the time of collection	Temp. ° C.	Date spawned	Days to reach maturity (stage V)
7/27/58	15	stage III	30.0 ± 1.0	8/4/58	6
12/13/58*	20	stages II and III	23.0 ± 1.0	1/9/59	26
8/28/58	8	stage VI	29.0 ± 1.0	9/29/58	35

* This sample was maintained at higher temperature when the natural habitat temperatures were approximately 14° C. The other two observations were done at approximately identical temperatures as those in the natural habitat during that period of the year.

color, with no differentiation between testicular and ovarian regions. The gonads were shrunken and flaccid with empty follicles. The changes in gonadal condition of bay scallops obtained from Alligator Harbor during 1957–1958 are shown in Table II.

Influence of temperature on gonad maturity

Scallops, 30–35 mm. long, obtained from their natural habitat during December when the water temperature was 14° C., were maintained in running sea water at two experimental temperatures. A control group was maintained at 14° C. and another batch was maintained at 23 ± 1.0° C. The only food available to the animals during this period was that which was circulating in the laboratory sea water system. Scallops maintained at the higher temperature reached stage IV in 26 days while the gonadal condition of those kept at 14° C. remained the same (Table III). The scallops which matured at the elevated temperature spawned successfully and the resultant larvae were cultured in the laboratory.

Observations on the reproductive cycle of animals from the field showed that most of the animals reached stage II or III by the beginning of winter. They remained in this condition throughout the cooler months (Table I) and resumed

TABLE IV

Average monthly temperature and salinity during 1957–1958 in Alligator Harbor, Florida

Month	Temperature ° C.	Salinity ⁰/₀₀
June, 1957	30.0	27.5
July	29.0	31.8
August	31.8	
February, 1958	8.9	
April	19.4	29.5
June	30.1	26.6
July	31.5	28.1
August	29.2	29.3
September	28.9	30.0
October	24.2	31.3
November	22.0	
December	13.6	32.4

development with the rising spring temperatures. Apparently low temperatures slowed maturation of gonads because population samples collected and maintained in the laboratory at elevated temperatures spawned successfully. Temperature and salinity data from Alligator Harbor region during 1957–1958 are shown in Table IV.

Spawning period

Observations on gonad condition of bay scallops obtained from their natural habitat (Table I) indicate that the spawning period commences in Alligator Harbor region at the beginning of August. Peak spawning takes place during August and September. Some minor spawning continues beyond September but this seems to add little to the population. A correlation of peak spawning period with temperature data in the study area suggests that the bay scallops began spawning when temperature decreased after a summer maximum, at least in 1958.

Effect of temperature on spawning

Spawning of bay scallops in the laboratory was obtained only when the animals were subjected first to increasing temperatures and then decreasing temperatures. In all other experimental conditions mentioned in the methods section, no spawning occurred. Addition of warm sea water and later cooling also induced spawning in most trials. It was not possible to induce spawning when the water temperature was increased to 30° C. without subsequent cooling. These laboratory observations on the effect of temperature on spawning are in agreement with the temperature conditions observed at the time of initiation of spawning period in nature. Apparently an initial gradual increase and a later decrease in temperatures are necessary to initiate spawning in bay scallops. Salinity does not vary widely in the study area. Laboratory observations also indicate that light conditions do not influence the spawning behavior in the bay scallops.

TABLE V

The effect of different maintenance temperatures on spawning temperatures of the bay scallops in the laboratory

Date spawned	No. animals spawned	Maintenance temperature ° C.	Increase in temperature ° C.	Spawning temperature ° C.	Nature of spawn
8/11/58	2	29.5	30.0	27.5	eggs
8/19/58	4	29.0	30.0	27.0	eggs
12/22/58	2	12.5	30.0	23.0	eggs
1/9/59	2	10.5	30.0	22.0	eggs
1/16/59	2	14.0	30.0	23.0	eggs
9/29/59	5	27.1	30.0	24.6	eggs, sperm
2/16/60	1	10.5	30.0	27.9	sperm
3/12/60	2	14.5	30.0	25.5	eggs

Spawning of bay scallops was partial in the laboratory. With only a few exceptions the spawn consisted of eggs only. When the animals liberated both sperm and eggs, there was a time lag between liberation of either eggs or sperm. Simultaneous liberation of both types of gametes was never observed in the laboratory-spawned scallops.

Laboratory spawning was induced during different months of a year. The animals used in these experiments were at different temperatures in the laboratory sea water prior to induction, with identical experimental procedures. The results of these observations are shown in Table V. The length of time and the temperatures at which the animals were in laboratory sea water tables before the experiments were not constant. The temperature at which the animals liberated gametes showed some interesting differences. The animals spawned during cooler months, in general liberated spawn at lower temperatures as compared with those from warmer months.

DISCUSSION

The changes in the gonad cycle of the bay scallop collected during different months of 1957–1958 showed that maturity is reached towards the end of July and spawning commences at the beginning of August in Alligator Harbor, Florida. The beginning of spawning coincides with decreasing temperatures following a summer maximum during 1958.

The development of gonads to maturity is slowed during winter months in nature. Animals brought into the laboratory in December and maintained at elevated temperatures developed to maturity in 26 days from the time of introduction into the sea water tables. This suggests that perhaps a time-temperature relation exists in gonadal development of bay scallops to maturity, once the gametogenesis has been initiated in their natural habitat. Scallops reared from eggs in the laboratory at 24.5 ± 0.5° C. failed to initiate gametogenesis at the age of 3–4 months while a comparable age group from natural habitat showed primary germ cells.

There are too few experimental temperature observations at which the scallops matured in the laboratory to warrant any concrete conclusions regarding the factors that control reproduction. However, these limited observations suggest that maturation of bay scallops may be controlled by exogenous factors after initiation of gametogenesis in their natural environment.

Laboratory spawning of scallops shows that an initial increasing and subsequent decreasing of temperatures stimulate liberation of gametes. Results obtained from spawning of scallops during different months of a year showed some interesting differences in the temperature at which they spawned. These experiments have to be done with more critically controlled conditions to find out if there is an effect of temperature acclimation on spawning. These observations on spawning in the laboratory indicate that spawning of bay scallops is not restricted to a particular period in the year or to a critical temperature. It appears that spawning is dependent on the maturation condition of the gonads and a favorable external stimulus. These observations suggest the possibility that spawning of bay scallops is not entirely operated by endogenous factors but by a combination of internal and external factors.

A comparison of the available information on reproduction of bay scallops from more northern geographical areas (Belding, 1910; Gutsell, 1930) with the results obtained for a Florida population show some differences in peak reproductive period, spawning behavior and the temperature requirements for maturation. Belding (1910) reported that the gonads of bay scallops in Massachusetts reach maturity and commence spawning towards the middle of June when the water temperatures are approximately 14–16° C. Gutsell (1930), working with Beaufort, North Carolina, populations, stated that spawning commenced in August. Observation on scallops at Beaufort during the fall of 1961 showed that they reach maturity early in fall and commence spawning. As already stated the Florida population of scallops commences spawning at the beginning of August. The spawning period in these three geographic populations is different, and also the spawning stimulus appears to be different, especially in Massachusetts and Florida. Belding (1910) reported that the bay scallops in Massachusetts commence spawning with increasing temperatures. Gutsell (1930) noted that the Beaufort population commences spawning with decreasing temperatures in fall. Laboratory and field observations in the present study indicate that the Florida population commences spawning with decreasing temperature after an initial maximum.

A comparison of these observations on latitudinal populations permits some generalizations on the subspecies from Atlantic and Gulf Coasts. The temperature requirements for maturation and spawning of bay scallops from south of Cape Hatteras appear to be higher than those from further north. Differences in spawning behavior and peak spawning period also occur between the northern and southern populations. Loosanoff (1952, 1956) and Korringa (1957) showed that populations of some species of bivalve mollusks from different geographical areas show variations in their temperature requirements for maturation and spawning. Transplanted latitudinal populations of bay scallops would enable testing these physiological differences for reproduction and their adaptive value.

SUMMARY

1. Subspecies *A. i. concentricus* from Alligator Harbor, Florida, reaches maturity towards the end of July when the summer temperatures are maximum and commences spawning early in August as the temperatures begin to decrease.

2. Winter temperatures slow gonadal development of a Florida population of scallops. During winter, scallops were stimulated to maturity at elevated temperature of 23.0 ± 1.0° C. in 26 days.

3. Matured scallops maintained in the laboratory spawned in response to elevated temperature during different months of the year.

4. The subspecies *A. i. irradians* from Massachusetts and *A. i. concentricus* from Florida and North Carolina show differences in temperature requirements for maturation and spawning. The subspecies north of Cape Hatteras reproduce at lower temperatures as compared with the southern subspecies. The spawning of both the subspecies does not overlap.

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ON THE ROLE OF THE LOFT, THE DISTANCE AND SITE OF RELEASE IN PIGEON HOMING (THE "CROSS-LOFT EXPERIMENT")

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THE PROBLEM

The idea of "map and compass" as an explanation for the homing orientation of pigeons was originally proposed by Kramer (1953a). The displaced pigeon, it was suggested, first determined its geographical position and then selected the home direction. This concept was inspired by Kramer's (1950, 1951, 1952) discovery of the sun compass in birds, a mechanism suitable for the selection and maintenance of directions. The actual operation of such a mechanism in the process of initial orientation of homing pigeons has subsequently been demonstrated (Schmidt-Koenig, 1958, 1960, 1961). The isolation of the sun compass as one constituent of the homing process supported Kramer's concept. But, although the idea of map and compass had been formulated years ago and though the discussion usually referred to this "map" as a literal representation of the geographical position of displacement, attempts to analyze its necessary components have not been made until recently (Schmidt-Koenig, 1960, 1961). It should be briefly reiterated that "map" means that the position of a displaced bird would have to be established by geographical or other coordinates. It would then be related to home in another step. Two divergent quantities would be involved: the direction and the distance from home. The directional part has been the subject of extensive considerations. However, after Kramer himself (1953b) had briefly touched on the role of distance, no evidence has become known dealing with the precise role of this parameter in the process of homing orientation. Matthews (1955) discussed distance in the light of his hypothesis, contributing some diagrams of poor initial orientation at less than 50 miles and of good initial orientation at more than 50 miles. Good initial orientation at less than 50 miles was also observed, however, and attributed to recognition of landmarks.

The distance from home can be thought of in the following way:

1. It may be directly taken into account by the bird's navigational system.
2. It may merely affect the accuracy of the bird's navigational system.
3. It may be irrelevant to the bird's navigational system. Mechanisms operating exclusively on directional information can be visualized as being relatively simple.

The present attempt to investigate the role of the distance is based on the following consideration: If a number of lofts are established in the shape of a symmetrical cross, with several kilometers' distance between lofts, the inhabitants of these lofts may be released from points along the extension of the axes of this cross. From points close to the cross, birds from certain lofts would face identical home directions but different distances, whereas birds from other lofts would face different directions but similar distances. Going farther away from the cross, these differences would diminish. It was the objective of the study reported here to investigate the effect of such a controlled diversity of parameters on homing within a range of less than 100 km.

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Experimental Design

In 1960, the Duke University colony was represented by two long-existing lofts, the Baucom loft and the Duke Forest lofts (Kramer, Pratt and St. Paul, 1958). Three more lofts were established in spring, 1960, to form a reasonably symmetrical cross (Fig. 1). The lofts were named according to their location within the cross as "center loft" (Baucom), "east loft" (Duke Forest) and so on.

Two release sites were selected along each of the axes of the cross. The first, short-distance site was located as close as possible to the cross, the second site at about three times the distance between the center loft and the first release site. The exact figures for bearings and distances may be taken from Table I.

Experimental Procedure

The three new lofts were stocked with youngsters from the Duke Forest and Baucom lofts which were, in turn, inhabited by birds from the "Duke University strain," originally founded (by J. G. Pratt) and eventually supplemented with birds obtained from local pigeon racers (*cf.* Schmidt-Koenig, 1963).

All naive birds were subjected to 3–4 exercise releases from various directions and 5–15 km. from home before they were used for critical cross-loft releases. Orientational pre-experience was kept about equal for all flocks, if necessary by supplementary exercise releases. All releases from the short-distance sites were performed between September and December, 1960, all releases from the longer-distance sites between February and May, 1961. The sequence of releases was designed to avoid successive releases from the same direction.

For releases, the birds were collected at night and driven to the release site the next morning inside a covered pick-up truck. At the release site, the birds were taken singly from covered crates and released with random alternations between birds from the various lofts. Each bird was followed with field glasses to

TABLE I

Detailed information on bearings and distances from the nine release sites to the various lofts; numerical account of the vector diagrams in Figure 1; homing success for all release sites and lofts. Underlining indicates data pooled from two (exceptionally three) releases. Brackets indicate distributions random at $p > 0.05$

Release site name (direction)	Loft	Home		Vanishing data Direction and length of mean vectors and sample size (as in Fig. 1)						Homing success Total of birds	
		distance km.	direction	α	a	n	α	a	n	released	lost
Caldwell School (N)	N	7.0	195°	*185°*	*.56*	*29*				51	4
	E	22.5	174°	*147°*	*.50*	*30*				43	6
	S	29.5	190°	*158°*	*.70*	*34*				37	4
	W	20.0	210°	*313°*	*.61*	*39*				43	3
	C	21.0	192°	*301°*	*.83*	*26*				39	2
Durham (E)	N	21.2	331°	*268°*	*.61*	*27*	144°	.52	14	34	6
	E	7.6	295°	*247°*	*.69*	*28*				31	7
	S	16.5	261°	*232°*	*.50*	*30*				34	2
	W	21.0	294°	*238°*	*.63*	*34*				49	2
	C	14.5	291°	*249°*	*.81*	*34*				34	4
Chapel Hill (S)	N	28.0	16°	*309°*	*.55*	*27*				36	4
	E	15.9	41°	*52°*	*.76*	*22*	*(87°)*	*.19*	*25*	63	4
	S	5.4	19°	*(241°)*	*.17*	*27*				36	1
	W	16.5	356°	*277°*	*.69*	*27*				28	4
	C	14.0	19°	*269°*	*.72*	*32*	191°	.62	17	52	8
Hillsboro (W)	N	17.0	61°	44°	.64	14	(57°)	.31	13	36	3
	E	19.6	113°	*123°*	*.65*	*28*	284°	.55	12	60	11
	S	16.3	149°	*165°*	*.75*	*28*	210°	.83	13	49	2
	W	6.5	112°	*111°*	*.91*	*38**				49	2
	C	13.0	118°	*242°*	*.66*	*33*				38	2
South Boston (N)	N	61.3	199°	*(291°)*	*.08*	*31*	194°	.71	10	54	11
	E	75.5	192°	*218°*	*.68*	*33*				38	4
	S	83.5	197°	*187°*	*.75*	*34**				43	8
	W	73.8	202°	*(297°)*	*.19*	*38**	274°	.58	11	59	5
	C	75.0	197°	*221°*	*.57*	*35*				39	8
Raleigh (E)	N	51.3	310°	*339°*	*.66*	*33*				35	7
	E	38.8	295°	298°	.56	17	(258°)	.41	14	37	5
	S	45.5	283°	*281°*	*.50*	*27*	(139°)	.12	15	45	6
	W	68.7	294°	*294°*	*.70*	*28*	1°	.74	13	45	6
	C	45.5	293°	*301°*	*.72*	*32*				37	3
Sanford (S)	N	67.8	17°	*318°*	*.69*	*31*				37	0
	E	54.9	27°	327°	.60	26	266°	.43	16	55	3
	S	45.5	18°	*322°*	*.55*	*27*				36	1
	W	55.5	13°	*331°*	*.81*	*29*				34	2
	C	54.0	19°	*325°*	*.60*	*36*				37	2

* Three releases.

TABLE I—(*Continued*)

Release site name (direction)	Loft	Home		Vanishing data Direction and length of mean vectors and sample size (as in Fig. 1)						Homing success Total of birds	
		distance km.	direction	α	a	n	α	a	n	released	lost
Burlington	N	40.0	96°	*(348°)*	*.11*	*27*				30	5
(W)	E	47.8	115°	*(145°)*	*.13*	*29*	205°	.60	21	54	8
	S	43.0	122°	*152°*	*.45*	*29*	*201°*	*.69*	*23*	57	5
	W	34.5	117°	*320°*	*.64*	*31*				32	4
	C	41.0	117°	*190°*	*.64*	*32*	283°	.75	16	75	6
				(309°)	.12	13					
Reidsville	N	73.0	105°	*(123°)*	*.30*	*23*				25	1
	E	81.5	115°	91°	.86	19	159°	.59	11	50	11
				(23°)	.27	12					
(W)	S	76.5	123°	*136°*	*.73*	24	(—)	.00	9	40	5
	W	68.4	116°	*146°*	*.61*	*29*	(167°)	.31	16	47	1
	C	75.0	116°	*129°*	*.46*	*27*				33	7

the vanishing point. The bearings at vanishing were recorded to the nearest 5° interval. The time elapsing between take-off and vanishing was recorded with a stop watch. Recording of homing performance, providing individual homing speeds, would have required five permanent welcomers for each releasing day, an expense beyond our budget. Instead, homing success, as established by the number of birds that did home, was recorded.

STATISTICAL DESIGN

As a standard procedure, two releases were performed from each site, each involving 15 to 20 (exceptionally more) birds from each loft. This meant the liberation of 75–100 birds within 5–8 hours. The vanishing bearings of the birds from each loft in each release were calculated to give α, the direction of the mean vector and a, its length (Gumbel, Greenwood and Durand, 1953). These quantities were then examined for randomness according to Durand and Greenwood (1958) and Greenwood and Durand (1955) and a graph derived from these authors (Schmidt-Koenig, 1961, appendix). The term, "release," subsequently applies to the data from birds of one loft obtained during one releasing day.

The following statistical procedure was adopted for experimenting and also maintained for graphical demonstration (Fig. 1; Table I). If the mean vectors of two releases (*i.e.*, at a given release site from birds of particular lofts) agreed by (a) either being both non-random ($p \leq 0.05$) and different from each other by not more than 35° [1], or if (b) both were random ($p > 0.05$), then the scores of the two releases were pooled and are given in Figure 1 with a double symbol (this applied, for example, to all releases from Caldwell School). If the two vectors either (c) were non-random (levels as above) but differed by more than 35°, or (d) were at random one time and non-random the other time, a third release was performed. Then, the scores of those two releases that met the requirements (a) and (b) were pooled and were plotted in Figure 1 with a double symbol, the other release given in a single symbol (*e.g.*, east loft and south loft at Hillsboro, north loft at South Boston). If all three disagreed, three single symbols were given (*e.g.*, east loft at Reidsville). It was further intended to end with about 30 vanishing scores for what may be termed the "preferred direction." However, a certain proportion of birds usually fails to yield vanishing scores either because the observer loses a few birds from sight before they reach the vanishing point or because some birds join stray pigeons or because some perch. In addition, the population of particular lofts sometimes was too low to afford 15–20 birds for a release. Thus, exceptionally four releases had to be performed, as may be seen from Figure 1 and Table I (*e.g.*, south loft and central loft at Burlington). Detailed information on vanishing data and on homing success may be taken from Table I.

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[1] At the time of the experiments, no specific test to discriminate between two circular samples was yet available; 35° was estimated to be reasonable.

The statistical analysis is, unfortunately, handicapped by the lack of certain methods specific for circular data. The approaches that were chosen for the various problems to be discussed in subsequent sections represent tentative solutions which, at the moment, seem to be the best available, and are certainly not grossly incorrect. The particular methods will be indicated in the text.

RESULTS

The first glance at Figure 1 reveals a puzzling diversity of headings. Differences according to loft membership seem to be prevalent. I shall, therefore, first focus on loft-specific differences.

1. *Vanishing data*

In 22 out of the grand total of 111 releases, the birds were distributed at random (at $p > 0.05$). The highest proportion of releases with random vanishing patterns was scored by the north loft with 7 out of 20 or 35% of the releases, the lowest proportion for the central loft with 1 out of 21 or 4.8% of the releases. The other lofts scored 26% (east), 17% (south), and 17% (west) out of 23, 23 and 24 releases, respectively. These rates are inhomogeneous at $p = 0.14$ but the rate of the center loft is different at $p = 0.02$ (χ^2-test) from that of the north loft. The rate of random releases was smaller (at $p = 0.055$; χ^2-test) for the short-distance sites, with 5 out of 47 releases, than the rate for the longer-distance sites, with 17 out of 64 releases (including Reidsville).

Eighteen times, the first two releases disagreed, six times for the east loft and 4, 3, 3, and 2 times for the south, west, north, and central loft, respectively. The third release also disagreed with any of the first two releases, one time each for the east and the center loft.

Drastically differing directional preferences characterize primarily the diagrams of the short-distance release sites. Pigeons whose home direction differed only by a few degrees or not at all headed into diverging, sometimes into opposite, directions. No consistency is to be observed between differences in distance and di-

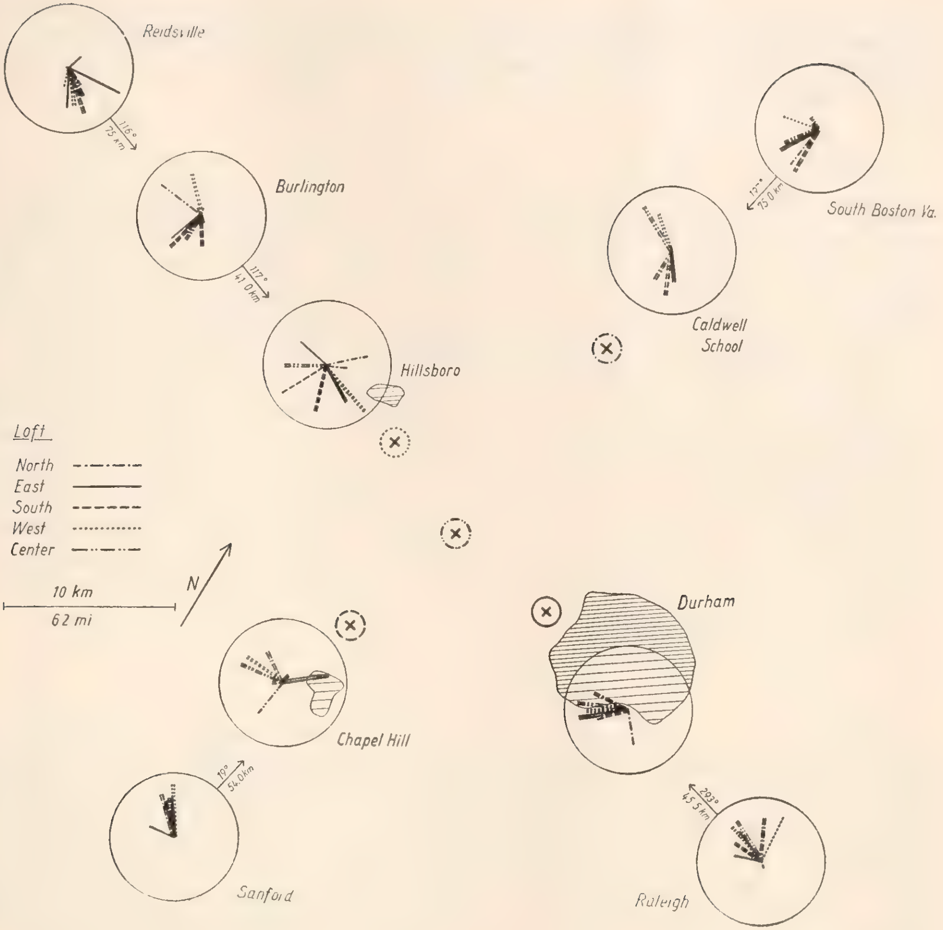
Reidsville
116°
75 km
Burlington
117°
41.0 km
South Boston Va.
12°
75.0 km
Caldwell School
Hillsboro
Loft
North
East
South
West
Center
10 km
6.2 mi
N
Durham
Chapel Hill
10°
54.0 km
Sanford
293°
45.5 km
Raleigh

FIGURE 1. Lofts and vanishing diagrams. The location of each loft is designated by a cross encircled by its symbol (according to the key). The diagrams from the four short-distance release sites are centered according to scale, those from the five longer-distance sites are referred to the center loft with proper indications of distance and bearing at the centrifugal arrow. The vectors are given by the respective loft symbol. The radius of each diagram corresponds to 1 (unit vector). The mathematical procedure is explained in the text. The precise quantities can be read from Table I.

rection from home and the vanishing pattern of the birds from the various lofts. Even qualitative directional differences of headings do not consistently agree with corresponding differences in home direction. The reader may easily select proper examples from Figure 1 and Table I, respectively.

Although the diversity was clearly reduced at the longer-distance sites, there were still some evidently diverging directional preferences. There is also some indication that the diversity and the reduction of diversity with increasing distance varied in different directions. The least tendency for diversity seemed to be from the east, then south, and north, finally the largest from the west. This relation is supported by the finding of an overall west tendency seemingly superimposed on all headings (Fig. 3). The relation between vanishing scores and the home direction is discussed more quantitatively below.

2. *Homing success*

The rate of losses was inhomogeneous at $p = 0.07$ (χ^2-test) for all lofts. However, significantly different rates ($p \leq 0.01$) between some lofts are established (north 11.8%, east 13.7%, south 9.0%, west 7.5%, center 10.9%; *cf.* Table I). Homing success is known to be much less sensitive a criterion than homing performance. There is good reason to assume that more striking differences would have been observed on the basis of individual homing speeds. Homing success from the longer-distance releases was not significantly different from that of the short-distance releases (Table I). This is easily explained since the orientational experience of the birds, as judged on the average number of releases performed, was much greater (10–20 releases) in the longer-distance releases than in the shorter-distance releases (6–12 releases). Homing performance of the Duke strain has been shown to improve steadily up to and perhaps beyond the twentieth release per bird (Schmidt-Koenig, 1963), while the tendency to scatter and homeward directedness of departures probably does not improve beyond about the eighth to tenth release (*op. cit.*).

Discussion

1. *The distance*

The original aim of this investigation was to find out whether the initial orientation of birds facing sizably different distances, but identical home directions, or vice versa, would differ in some typical fashion. Considering the variation among the lofts and within the range tested (below 100 km.), this was clearly not so. But indications that the distance may enter into the mechanism of navigation emerge when larger dimensions than those few kilometers difference between the lofts are considered. The qualitative impression from Figure 1, that strongly diverging headings diminish with increasing distance from home, may be turned into a more quantitative consideration. In Figure 2 all vanishing scores were calculated to yield h′ the homeward component. This statistic is calculated as $h = a.\cos(\alpha\text{-}\beta)$, where a is the length of the mean vector α of a vanishing diagram and β the home direction. The prime symbolizes quantities obtained upon pooling data from more than one release. Although the varying sample size and the absence of specific statistical tests render this a rather rough approach, we may note in Figure 2 some association between accuracy of headings and distance. Homeward directedness seems to improve with increasing distance insofar as violently deviating tendencies (exhibited primarily by the center- and the west-loft birds) do not occur any more. But there is no or not much improvement of accuracy within the home half of the circle ($h' > 0.0$), along with a tentative indication for an increase of random distributions for the longer distance sites (35–84 km.). One more restriction has to be made: due to the design of the experiment, the birds' orientational experience also grew with increasing distance. The lowest level of experience of any individual participant in the short-distance releases was

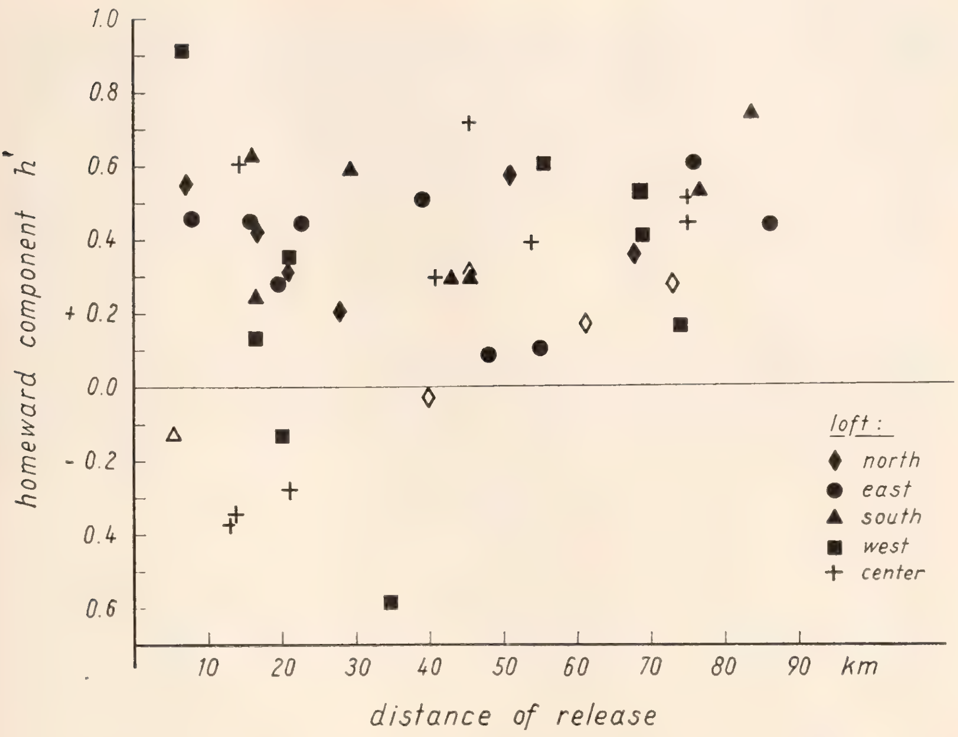

FIGURE 2. The homeward component h′ summarizing all vanishing scores per loft at each release site. Open symbols stand for distributions random at $p > 0.05$. The homeward component is positive if the mean vector of a distribution falls within the homeward half of the circle; if a mean vector falls into the other half, the homeward component becomes negative. The value $h = +1.0$ would indicate that vanishing points uniformly coincided with the home direction, $h = -1.0$ would mean all birds vanished at 180° from home. A value of $h \cong 0.0$ may indicate either that vanishing points clustered around ± 90° from the home direction or that they were distributed approximately at random.

never lower than the fourth release. In the longer-distance release sites the levels of experience were accordingly higher. Thus, the conclusion remains tentative for several reasons.

As an indication for the variance of headings, a′ was computed for the same data for which h′ was given in Figure 2. There seemed to be no reduction of scatter with increasing distance (no Figure).

2. *The loft*

Several authors (Pratt, 1955; Kramer, Pratt and St. Paul, 1956, 1958; Pratt and Wallraff, 1958; Graue and Pratt, 1959; Hoffmann, 1959) present or discuss indications that even consanguineous birds may differ in homing performance when settled at different geographical locations. The present study (a) extends such findings on other portions of the homing process and (b) indicates differences between lofts just a few kilometers apart from each other. The evidence of the above-mentioned publications suggested the differences were either largely due to some gross features, either topographical factors as presence or absence of mountain ranges, water bodies and the like, or to the orientational clues varying in strength or kind. Wallraff (1960a) concluded from extensive correlation analyses of variations in the performance of Wilhelmshaven colony birds that these variations, at least to some extent, were due to large scale meteorological and geophysical fluctuations. He further concluded that these variables interfere directly with the clues utilized by the birds. Such an interpretation appears to be hardly applicable to the differences and variations observed in the cross-loft experiment. They were certainly not characteristics of the general area, they affected one or two, never all lofts on a given release day. Certain release sites or even general directions seemed to be more sensitive than others in reflecting differences and variations in differences. Already Kramer, Pratt and St. Paul (1956, 1958) have come across indications for differences in the performance of the east (Duke Forest) loft, the center (Baucom) loft and the Nation Ave. loft birds near

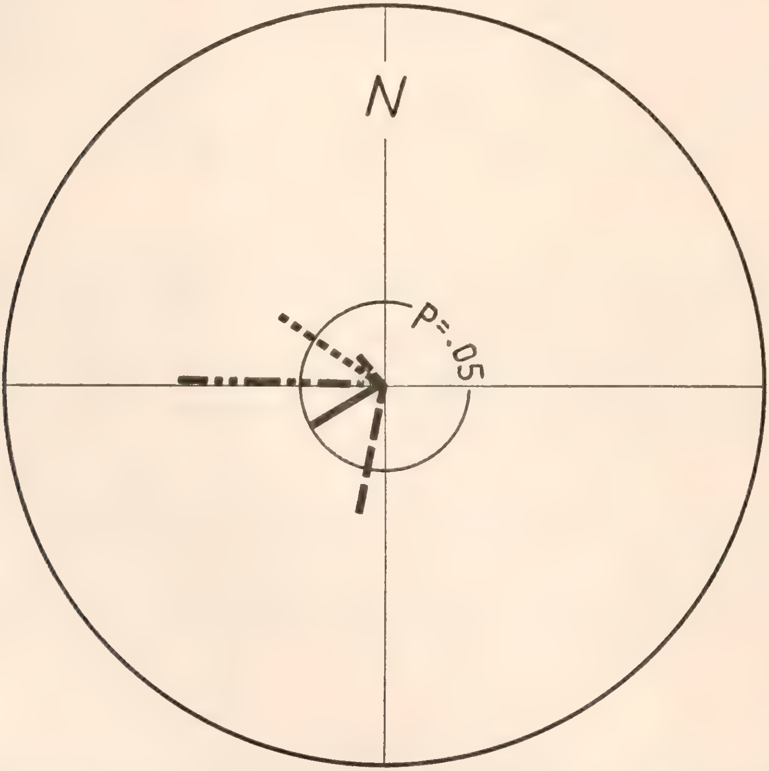

FIGURE 3. General directional tendency for each loft. To obtain a fairly symmetrical account, the first two releases of each loft at the eight main sites (exclusive of Reidsville) have been pooled. Each vector represents 230–258 vanishing scores. The radius of the circle corresponds to 1 (unit vector). The 5% level for randomness, extrapolated from Durand and Greenwood (1958, Figure 1 upper diagram) for the mean sample size (245), is indicated.

Durham. The evidence, however, was not sufficiently striking to conclude there were loft-specific factors. These authors realized that the concept of "directional differences" may have to be abandoned. Wallraff (1959a, 1959b; 1960a, 1960b) focused entirely on "local effects" for deviations of headings from the home direction and their variations. Graue and Pratt (1959) suggested not some feature at the release site but the direction of flight from a given point as contributing at least a component to the "local effect." Thus, there is a line of observations that leads to the view that the bird's goal may contribute a component to the unexplained variations in homing.

3. *Other aspects*

There is still a chance that a general directional tendency is superimposed on headings. For Wilhelmshaven, a north to northwest trend is well known. It is probably due to the polarized release pattern lacking flights from the north. No account is available for other loft sites in Germany. Matthews' (1955) data suggest no general directional tendency in long-distance release (up to 170 km.) in England. Graue and Pratt's data from a symmetrical release pattern on the north-south axis in California show a westerly component. In order to have a fairly, though not strictly, symmetrical total of vanishing scores for the Durham area, the data from the first two releases of the cross-loft series at the eight symmetrical release sites (exclusive of Reidsville) have been pooled for each loft. The mean vectors are given in Figure 3. A general westerly trend is evident; however, loft-specific differences again seem to prevail. The scores of the north loft are at random ($p \approx 0.1$), those of the east loft are at the edge of significance ($p = 0.05$). This may mean that the birds tended either (a) to random vanishing, which obtains for the north loft, or (b) to more homeward directed departures, which obtains for the east loft. The question of general trends possibly superimposed on headings, despite symmetrical release patterns, will be more thoroughly discussed in another paper on the basis of more detailed data from North Carolina and other areas.

SUMMARY

1. Five lofts which formed a symmetrical cross, with several kilometers' distance between the lofts, were established. Releases were performed with birds from all lofts along the extensions of the axes of this cross. From points close to the cross, birds from certain lofts would face identical home directions but different distances whereas birds from other lofts would face different directions but similar distances. The effect of such controlled interrelations upon initial orientation and homing success below 100 km. was studied.

2. A diversity of directional preferences, but no consistency between the variations in distance and direction from home and direction of headings, was found.

3. A loft-specific basis for the differences and changes in vanishing behavior and homing success is suggested. Its cause is unknown. Previously suggested explanations, such as large scale topographical, geophysical, and meteorological factors and variations, are hardly applicable to these observations.

4. There may be some improvement of homeward directedness of headings

with increasing distance; however, random vanishing patterns were observed up to the longest distances tested (84 km.).

5. A slight general west tendency, apparently modified by loft-specific components, was observed for the Durham area.

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INCORPORATION OF LABELED VALINE INTO THE PROTEINS OF THE CECROPIA SILKWORM[1]

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The diapausing pupal silkmoth has "one of the lowest metabolic rates recorded for any aerobic animal" (Harvey and Williams, 1961, p. 82). Metabolism can be accelerated (1) in the presence of the hormone ecdyson, either exogenous or endogenous, or (2) following injury to the animal. In both cases the animal consumes more oxygen (Harvey and Williams, 1961; Schneiderman and Williams, 1954), synthesizes cytochromes (Shappirio, 1958, 1960), and forms proteins of both blood (Telfer and Williams, 1960) and midgut (Laufer, 1960) at an increased rate.

The consequences of this increased activity are very different in the two cases. Ecdyson stimulates the pupa to develop into an adult moth; injury leads only to wound repair of the pupa. During development, proteins are synthesized in all of the tissues which metamorphose into the organs of the adult; following injury, proteins *appear* to be synthesized principally in those tissues involved in repair, such as blood and epidermis. The present study was undertaken to characterize more fully the differences in the physiology of development *vis a vis* the physiology of wound repair. Specifically, this paper describes the results of experiments in which the incorporation of valine-1-C^{14} into the proteins of various tissues in the two conditions was studied.

Valine-1-C^{14} was used in these experiments because, at least in the metazoans in which the metabolism of valine has been studied (Kinnory *et al.*, 1955; Robinson *et al.*, 1957), the carboxy carbon is lost as CO_2 early in the degradation. Consequently, the radioactivity of a protein isolated from an animal following an injection of valine-1-C^{14} is likely to represent valine, as such.

Ultimately, the proteins which are synthesized during the pupal-adult transformation must be characterized. A first step in this direction was taken by fractionating a tissue and determining the incorporation of radioactive amino acid into nuclear, mitochondrial, microsomal, and soluble proteins. For these experiments thoracic muscle was used at the time when the rate of protein synthesis was at a maximum, so that the specific activity of the protein fractions would be high. The time of maximal synthesis was determined by measuring, from the onset of the development of the thoracic muscles until adult emergence, the concentration of cytochrome *c*. The recent discovery of methods for extracting cytochrome *c* from insect tissue (Tuppy, 1957) greatly facilitated these experiments.

Some of the results have been reported previously (Skinner, 1960).

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MATERIALS AND METHODS

1. *Selection and maintenance of animals*

The Cecropia silkworm, *Platysamia cecropia*, hatches from the egg as a larva, undergoes four larval molts followed by a pupal molt; the pupa then enters the diapause stage.

Female pupae were selected from populations reared locally on wild cherry trees. Additional pupae were made available through the kindness of Dr. C. M. Williams. Development was induced by bringing the animals to 25° after ten weeks of chilling at 6°. Stages of development were recognized from the criteria of Schneiderman and Williams (1954).

In order to stabilize pupae in permanent diapause, their brains were removed (Williams, 1946). After the operation, the pupae were maintained at 25° until use. All brainless pupae used in these experiments had been operated on at least 10 months previously; thus, none exhibited any characteristics of "injury metabolism" (Harvey and Williams, 1961; Schneiderman and Williams, 1953).

DL-valine-1-C^{14} was obtained from the New England Nuclear Corporation. The insect Ringer's solution was that of Ephrussi and Beadle (1936). Ecdyson was a purified preparation (Butenandt and Karlson, 1954) kindly presented to Dr. Gerard R. Wyatt by Dr. Karlson.

2. *Injection and surgery*

Before injections of valine-1-C^{14} or ecdyson and subsequent surgery, animals were anesthetized with CO_2. Unoperated pupae were injected via the dorsal vessel into the third abdominal segment, brainless diapausing pupae through their facial windows, and injured animals in the head region, just caudad to the wound. For the study of the effects of injury, the first pair of legs and the facial region were removed from debrained pupae. The wound was covered by a plastic window held in place by wax. Adults used for *in vitro* protein synthesis studies were immobilized by the removal of appendages. The injuries were sealed with wax.

When the incorporation of amino acid was to be measured, 0.43 μmole of DL-valine-1-C^{14} (specific activity 6 μc./μmole) was injected in a volume of 0.04 ml. insect Ringer's solution.

3. *Preparation of tissues*

(A) Epithelium: the wings, delicate, single-layered sacs of epithelium, were cut along their edges and squeezed together to force out blood. They were then rinsed several times by sucking up iced Ringer's solution into the epithelial sac and expelling it. Undoubtedly, some blood remained within the wings. (B) Midgut: the midgut was removed, slit along its length, cleaned of its contents and rinsed several times in iced Ringer's solution. (C) Muscle: the thorax was cut open along the mid-ventral line, freed of fat body and connective tissue, rinsed several times in iced Ringer's solution, blotted on hard-surface filter paper, and minced and transferred to 5% TCA. The exoskeleton was removed by centrifugation from 0.4 NaOH, one of the steps of the washing procedure used in preparing the precipitated proteins for counting. (D) Eggs: eggs were freed from the ovarioles and were rinsed several times in iced Ringer's solution and blotted to remove blood. (E) Fat body: the fat body was rinsed in iced Ringer's solution and blotted. (F) Blood: blood expelled from a puncture in one antenna was collected in cold 2-ml. test tubes. It was kept cold to prevent tyrosinase activity. Cells and any tissue contaminants were sedimented from the samples by centrifugation in the cold at 7000 × g for 10 minutes.

4. *Precipitation and preparation of proteins for counting*

The tissues were homogenized in 5% trichloracetic acid (TCA). They were then washed once with cold TCA and once with hot (90° C.) TCA to remove nucleic acids. The proteins were then solubilized in 0.4 N NaOH in the presence of unlabeled valine (10 mg./ml.) to facilitate the exchange of any adsorbed radioactive valine. After standing at room temperature for 30–60 minutes, the proteins were re-precipitated with TCA. Lipids were then extracted in one warm (70° C.) ethanol-ether (3:1 v/v) wash, and two ether washes (McLean *et al.*, 1958). From 0.5 to 3 mg. of the dried protein powder which is obtained by this washing procedure was plated on tared aluminum planchets which were weighed and then were counted in a gas flow windowless counter with a background of 15 cpm. Specific activity (SA) is defined as counts per minute per mg. protein.

5. *Determination of cytochrome c*

Thoraces of developing adults were dipped in melted wax which was allowed to harden and was then peeled off with scales attached. The thoraces were cut open along the mid-ventral line, freed of fat body and connective tissue, rinsed in Ringer's solution to remove blood, blotted on hard-surface filter paper, weighed and ground in 1 or 2 ml. of 0.7% sodium dithionite (Tuppy, 1957). The suspensions were kept at room temperature for five hours, after which time they were frozen to insure cell lysis and complete extraction of cytochrome *c*. After thawing, the extract was clarified by centrifuging for 10 to 20 minutes at 18,000 × g in the high-speed head of a refrigerated International centrifuge. Light absorbancy was measured at 550 mμ in a Beckman DU spectrophotometer. The cytochrome *c*, extracted in the reduced state in the presence of the dithionite, was oxidized by the addition of potassium ferricyanide and the absorption at 550 mμ again measured. The cytochrome *c* content was calculated, using the extinction coefficient of horse heart cytochrome *c* at this wave-length (Margoliash *et al.*, 1959a, 1959b).

6. *Preparation of subcellular components of flight muscle*

Anesthetized animals were placed on ice and taken to the cold room where their thoraces were prepared as for extraction of cytochrome *c*. The medium used throughout contained 0.3 M sucrose, and 0.003 M Versene, adjusted to pH 7.4.

The thoraces were minced with scissors, placed in a mortar with a few ml. of medium and gently crushed. The tissue suspension was strained by suction through a Buchner funnel to remove pieces of cuticle, and then filtered through cheesecloth. The tissue residue was scraped from the cheesecloth, and returned to the mortar. The whole process was repeated twice. The three filtrates were set aside for collection of sarcosomes and microsomes.

The tissue residue was ground in medium in a glass homogenizer to free the remaining myofibrils and to rupture the remaining sarcosomes. The myofibrils were collected by filtering through hard-surface filter paper (SS-589), and were washed with medium until the filtrate was colorless. They were scraped from the paper, suspended in 10 ml. of medium and sedimented by centrifuging at 200 × *g* for two minutes at 0°. The remaining pieces of connective tissue floated on the supernatant fluid and were discarded with it. The myofibril pellet was suspended and centrifuged again.

The filtrate obtained from the crushed tissue was centrifuged at 200 × *g* for two minutes to remove any myofibrils. It was then centrifuged at 1100 × *g* for 30 minutes to collect sarcosomes. A second smaller crop of sarcosomes was collected by re-centrifuging the supernatant at 1100 × *g* for 30 minutes. The pellets were re-suspended, pooled and washed once by centrifugation with medium. The original supernatant, free of sarcosomes, was centrifuged for one hour at 100,000 × *g* to collect microsomes (Edwards *et al.*, 1956). The final supernatant was the so-called soluble fraction. The proteins of each fraction were precipitated with TCA, final concentration 5%, and were washed and plated as described above.

7. *Measurement of oxygen consumption*

Oxygen consumption was measured manometrically in modified Warburg flasks described by Schneiderman and Williams (1953).

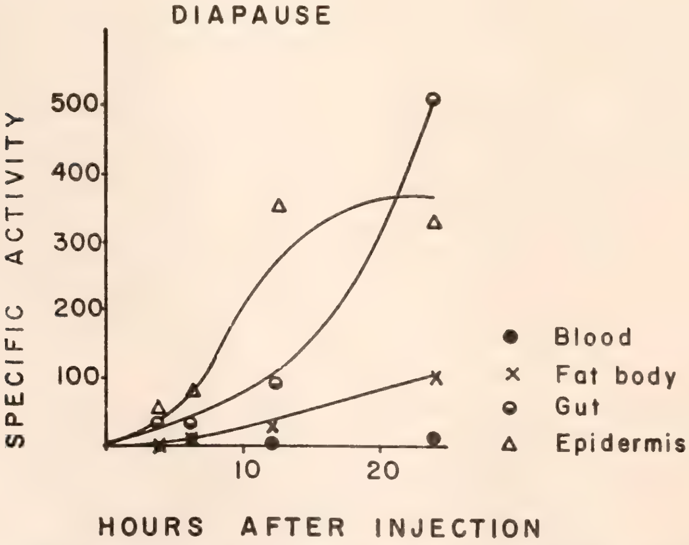

FIGURE 1. Sixteen brainless, diapausing pupae, each injected with 2.6 μcuries valine-1-C^{14}. Tissues removed from 4 animals at 4, 6, 12 and 24 hours after injection. Proteins precipitated, washed, plated and radioactivity measured. Specific activity equals counts per minute per mg. protein.

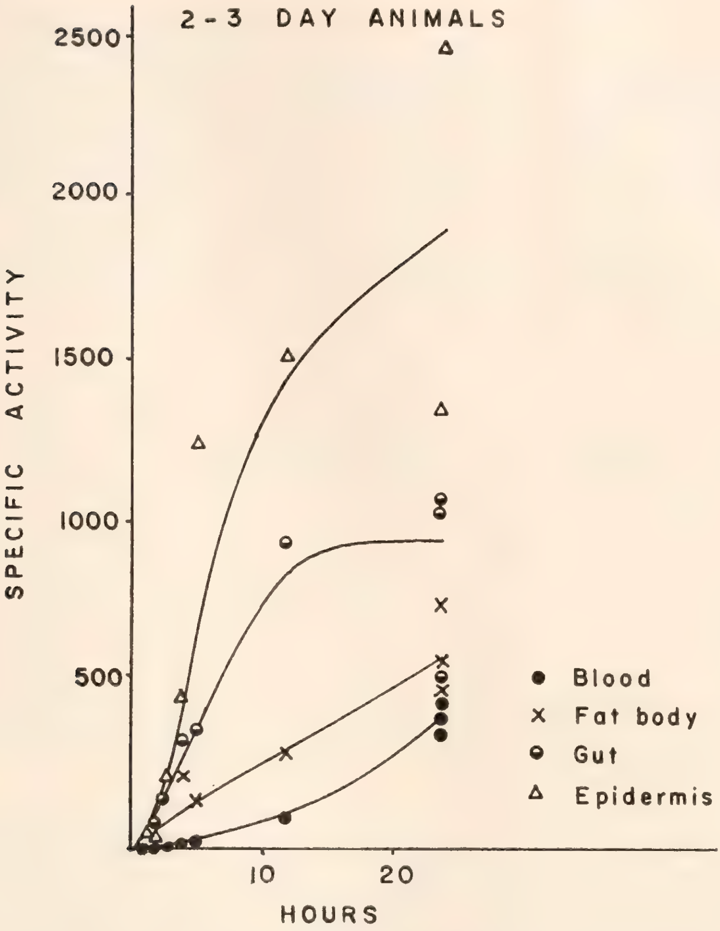

FIGURE 2. Thirty-one animals on the second or third day after initiation of adult development, each injected with 2.6 μcuries valine-1-C^{14}. Tissues removed from series of animals at times indicated. Proteins precipitated, washed, plated and radioactivity measured.

RESULTS

1. *Incorporation of valine-1-C^{14} into diapausing animals*

The results of an experiment with debrained diapausing pupae are assembled in Figure 1. The specific activity of the blood was very low, though of the same order of magnitude as found previously (Telfer and Williams, 1960). The rates of incorporation into proteins of the fat body, epidermis and midgut were approximately 30, 50 and 70 times as high, respectively.

2. *Incorporation of valine-1-C^{14} into the developing adult*

Two or three days after the initiation of adult development, there is a marked increase in the incorporation of labeled valine into the proteins of all tissues (Fig.

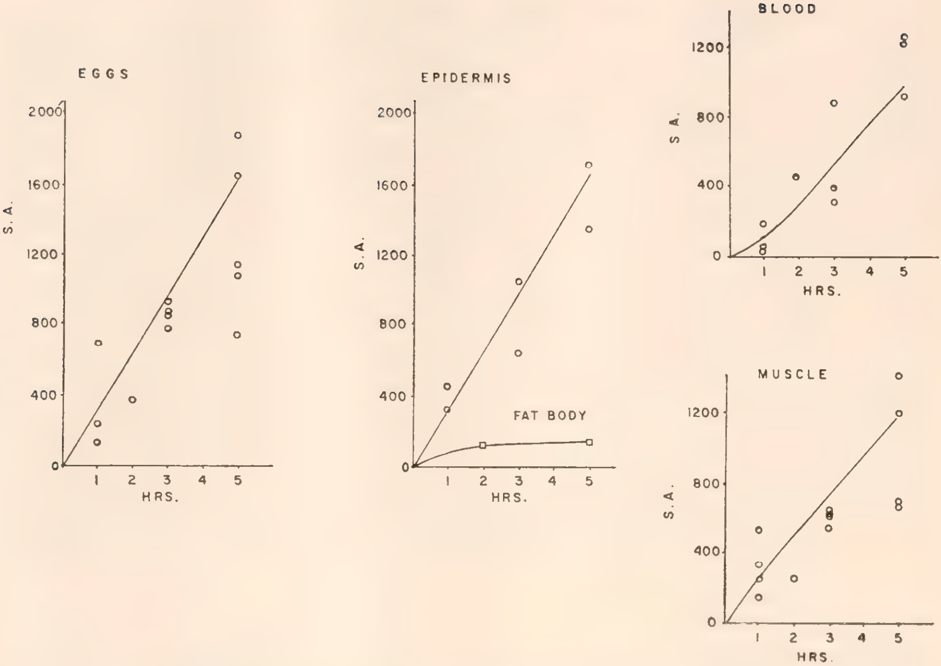

FIGURE 3. Thirteen animals on the seventeenth or nineteenth day of adult development, each injected with 2.6 μcuries valine-1-C^{14}. Tissues removed from groups of animals at times indicated. Proteins precipitated, washed, plated and radioactivity counted. □ = fat body; other tissues as indicated on graphs.

2). This increase is approximately 2-fold in the midgut, 5-fold in both fat body and epidermis, and, after an initial lag period, 200-fold in blood.

By the seventeenth day of development, there was a further increase in incorporation in some tissues (Fig. 3). The greatest increase was into blood proteins, which were 15 times more highly labeled than those of the two- or three-day-old animals. It should be noted that the fat body was no longer able to effect the incorporation of amino acids. The midgut has practically disappeared at this time; thus, it was not sampled. However, eggs and thoracic muscle, two newly differentiated tissues, had incorporated large amounts of valine.

3. *Incorporation of labeled valine into the adult*

The blood volume of adults 8 days after emergence is too small to be sampled, as are also remnants of gut and fat body. The two tissues available in animals of this age are eggs and thoracic muscle.

Only two adults were examined, and although the variability between results was great, there was a measurable incorporation of valine into somatic proteins and a very rapid incorporation into egg proteins. The decreased circulation of the small volume of blood which limits the supply of amino acids to the various tissues makes incorporation studies on adult moths technically difficult.

4. *Effect of injury on incorporation*

The oxygen consumption of four debrained animals was followed for a two-hour period. After it was established that the Q_{O_2}'s of the animals were at the low level characteristic of diapause (12 to 28 μl. O_2/gm. animal/hour (Schneiderman and Williams, 1953)) each animal was injured. Oxygen consumption was measured for two days while "injury metabolism" (Harvey and Williams, 1961; Schneiderman and Williams, 1953) developed. When their Q_{O_2}'s reached 45 to 90 μl. O_2/gm. animal/hour, the animals were injected with labeled valine and sacrificed in pairs: one after 3.5 hours, the other after 24 hours. As can be seen in Table I, the uptake of labeled valine by all tissues was greatly increased following injury.

TABLE I

Incorporation of valine-1-C^{14} into proteins of diapausing pupae after injury

Time (hours)	Specific activity			
	Tissue			
	Blood	Fat body	Gut	Epithelium
3.5	14	105	142	266
	(0)*	(0)	(40)	(50)
	30	97	102	157
24	340	518	794	3150
	(2)	(100)	(500)	(350)
	350	490	674	7180

* Figures in parentheses are the specific activities of uninjured diapausing pupae.

Four brainless diapausing pupae injured by removal of cuticle in the facial region; 48 hours after the injury, 2.6 μcuries valine-1-C^{14} injected into each animal. Two animals sacrificed 3.5 hours after injection; two others 24 hours after injection. Proteins precipitated, washed, plated, radioactivity measured.

5. *Protein synthesis of diapausing pupae after the administration of ecdyson*

The oxygen consumption of a group of debrained animals was measured. Six animals with Q_{O_2}'s around 30 were selected. Two of the six (control 1, Fig. 4) received no initial injection. Two others (control 2, Fig. 4) were injected with 0.04 ml. of 10% methanol in insect Ringer's solution; the other two were injected with 4000 "Calliphora units" (Butenandt and Karlson, 1954) of purified ecdyson in 0.04 ml. of 10% methanol in insect Ringer's solution. Between 16½ and 17 hours later, all six received the usual dose of labeled valine. Oxygen consumption was followed at intervals throughout this period. Between 25 and 26 hours after the first injections, the animals were examined for detachment of the epidermis in the region of the forelimbs—one of the first signs of development (Schneiderman and Williams, 1954). Only in those animals which had received ecdyson had the epithelium become so detached; the epithelium of the other animals was still firmly attached.

A glance at Figure 4 shows that though the injection of ecdyson greatly increased the amount of labeled amino acid incorporated into protein, its effect was not much greater than the effect of injury inflicted by injection of Ringer's solution (compare control 2 with the ecdyson animals). The specific activity of the blood proteins of the two pairs of animals which received injections prior to labeled valine (control and ecdyson animals) was only slightly higher than that of the pair which received no prior "injury" (control 1) due to the first injection. On the other hand, two to nine times as much labeled amino acid was incorporated into the other three tissues of the "injured" and "ecdyson" animals as into those of the uninjured animals.

6. *Cytochrome c concentration in thoracic muscles*

The thoracic muscles begin developing 8 to 10 days after initiation of adult development (Schneiderman and Williams, 1953). However, in Figure 5 it can be seen that with the present methods of extraction and spectrophotometry at room temperature, cytochrome *c* was not detected until the seventeenth day of adult development. From the seventeenth day of development until the time of adult emergence, the concentration increased greatly. Therefore, the radioactivity of subcellular fractions of thoracic muscle was measured in animals on the seventeenth and twenty-first days of adult development.

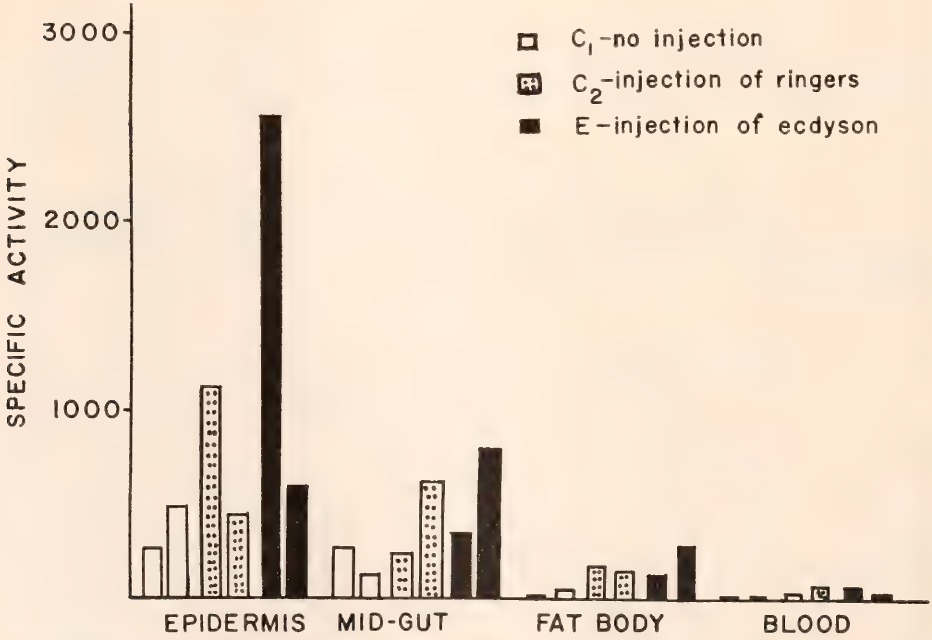

FIGURE 4. Six brainless, diapausing pupae, two of which (C_1) were not injected; two of which (C_2) were injected with 10% methanol in Ringer's solution; and two of which (E) were injected with 4000 Calliphora units of ecdyson. Sixteen and one-half to 17 hours later, all 6 were injected with 2.6 μcuries valine-1-C^{14}. All were sacrificed and their tissues removed 8 to 9 hours later. Proteins precipitated, washed, plated and radioactivity measured.

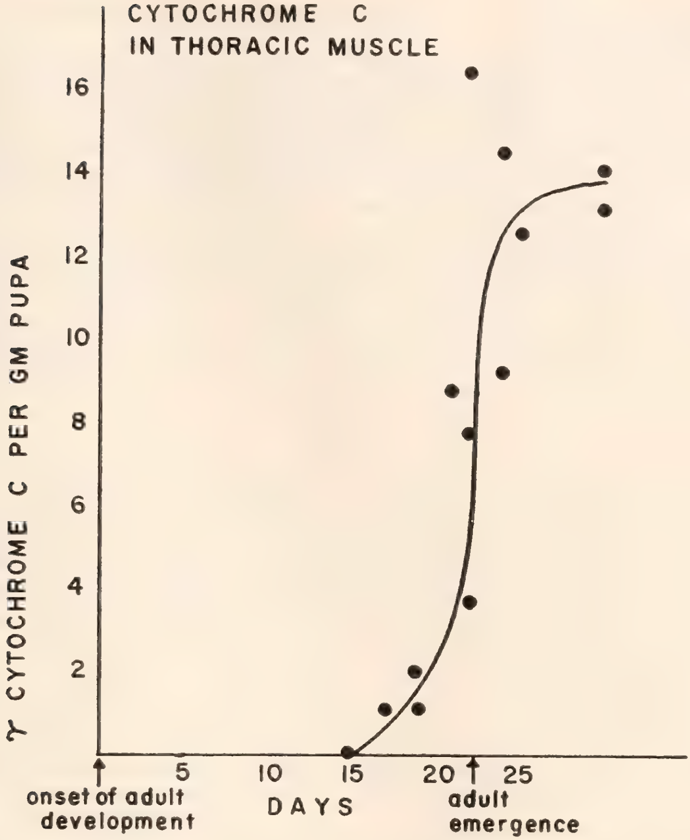

FIGURE 5. Cytochrome *c* extracted from a series of developing adults at the times indicated. Using extinction coefficient for beef heart cytochrome *c*, amount of cytochrome contained in each animal calculated and expressed as γ per gram fresh weight (animals weighed on the first day of adult development).

TABLE II

Incorporation of valine-1-C^{14} into subcellular fractions of thoracic muscle

Cell fraction	Expt. 1: 17-day animals	Expt. 2: 21-day animals
	Specific activity	
Microsomes	911	566
Myofibrils	766	320
Soluble	678	385
Sarcosomes	604	343

Four animals on the seventeenth day of adult development (Expt. 1) and eight animals on the twenty-first day of adult development (Expt. 2) each injected with 2.6 μcuries valine-1-C^{14}. Two hours after the injection, thoraces removed, excised muscle pooled and fractionated as described in text. Proteins of each fraction precipitated, washed, plated and radioactivity measured.

7. *Differential incorporation of radioactive valine into subcellular fractions of thoracic muscle*

The incorporation of labeled valine into the components of thoracic muscle was compared in several experiments. Groups of 4 to 8 animals were sacrificed two hours after injection of labeled valine. The pooled thoracic muscle was fractionated into sarcosomes, microsomes, myofibrils and soluble system as described above. Microscopic examination of the sarcosome preparations revealed their homogeneity. The final myofibril preparation contained some undisrupted sarcosomes.

The data in Table II indicate that in each case the microsomes have the highest specific activity, while the specific activity of the myofibrils, sarcosomes and soluble system are similar to one another. The specific activities of the samples from the 21-day-old animals are considerably lower than those from the 17-day animals.

DISCUSSION

The incorporation of labeled valine into proteins at different stages of development

All of the tissues of the diapausing pupae which were studied incorporated amino acids into their proteins, though the labeling of the blood proteins was slight. Within the first few days after initiation of adult development, incorporation rates of valine are increased three to five times into epithelium, mid-gut, and fat body, and several hundred times in blood.

In the epithelium the rate of incorporation remains constant in the late stages of development. In contrast, several days before adult emergence, the rate of labeling of blood proteins increases further, while that of fat body proteins decreases sharply. This shows that during development there is not a general over-all increase in amino acid incorporation but that the response to the hormone depends on the target tissue. Eggs which begin to form during the second week of adult development (Telfer, 1960) incorporate a large amount of valine. Indeed, the egg proteins of an eight-day-old adult had the highest specific activity of any tissue at any stage.

The synthesis of mid-gut proteins is high, even during diapause. The gut appears to synthesize numerous hydrolytic proteins (Laufer, 1960) which may be needed even by the diapausing animal. The increased formation of mid-gut proteins during the first few days of adult development may be a measure of the synthesis of additional hydrolytic enzymes responsible for breaking down pupal tissues.

As in the experiments of Telfer and Williams (1960), the rate of incorporation of radioactive amino acid into blood proteins of diapausing pupae was very low. The negligible incorporation into blood proteins, compared with other tissues of the diapausing pupa, is consistent with the generally accepted role of the blood as a storage organ. Hence, in diapause certain functional proteins of active tissues may be replaced but the formation of storage protein is negligible.

At later stages in development, when the rate of protein synthesis in all tissues is much faster, there is also an increase in the radioactivity of the blood proteins. Such incorporation is always preceded by a lag period of several hours. Probably, blood proteins are synthesized within cells from which they are then secreted.

The effect of ecdyson and injury on protein synthesis

Isolated abdomens of *Cecropia* pupae develop into adult abdomens after the injection of 20 γ (2667 Calliphora units) of crystalline ecdyson (Williams, 1954). The dosage of ecdyson used in the present experiments (4000 Calliphora units per animal) caused the initiation of adult development, as evidenced by retraction of the pupal cuticle (Schneiderman and Williams, 1954).

It was not surprising to find that the injection of ecdyson increases amino acid incorporation to the levels characteristic of the developing adult, since we believe that we are merely supplying the usual hormone from an exogenous source. However, it is somewhat unexpected to find that amino acid incorporation in all of the tissues studied is also increased by an injury to the pupa and that the increases are indistinguishable from the early stages of adult development.

This result emphasizes the fact that ecdyson release has two effects on a diapausing animal: (1) to increase metabolism and (2) to reroute metabolic pathways toward growth and differentiation. Injury can duplicate the first effect, but not the second.

To understand development requires more than the assay of overall metabolism. Future experiments must take into account the synthesis of specific molecules. One approach might be to study amino acid incorporation into specific sub-cellular fractions. The evidence presented here (Table II) indicates that amino acids are being incorporated into all cell fractions; thus, it must be concluded that no single type of cell component is being synthesized to the exclusion of all others.

The author wishes to thank Dr. Melvin V. Simpson, and Dr. Gerard R. Wyatt, in whose laboratory this work was done, for helpful discussions about the work presented, and Dr. William G. Van der Kloot for critical reading of the manuscript.

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BIOLUMINESCENT DINOFLAGELLATES[1, 2]

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From the days of the earliest voyages, men have observed the sparkling luminescence of the sea. That each fleck of light was usually the flash from a single creature of minute size rather than the phosphorescence of a chemical substance was known before the end of the eighteenth century (Harvey, 1952, 1957). The light-emitting properties of some of the dinoflagellates became apparent because these organisms were on occasion so very plentiful. One of the earliest to be recognized was *Noctiluca* (Baker, 1753; de Quatrefages, 1850) because of its large size and the great brightness of its luminescence, as well as its common occurrence along the shores of Europe. The "red tides" of *Gonyaulax polyedra* on the west coast of the United States and the extremely bright luminescence which accompanied them led to the identification of this organism as luminescent (Kofoid, 1911). A number of investigators have looked at luminescent sea water in the laboratory to try to identify the organism responsible (Dahlgren, 1924). The results of these studies have not always been definitive since the delicate organisms may have been in poor condition. Although culture of the dinoflagellates became possible following the discovery of their auxotrophy, observations of luminescence in cultures have seldom been reported, perhaps because most cultures were grown in continuous bright light, a condition which, in *Gonyaulax* at least, very much reduces the intensity of light emission. The relatively few species, the luminescence of which has already been well established, are given in Table I.

Since the author had at her disposal a photomultiplier photometer arranged for the measurement of the luminescence of the dinoflagellates (Sweeney, Haxo and Hastings, 1959) it seemed worth while to examine the common dinoflagellates of the coast of Southern California to determine which were luminescent and, perhaps equally interesting, which were not.

MATERIALS AND METHODS

The dinoflagellates to be tested were collected from the ocean off Scripps Institution of Oceanography at La Jolla, California. Water samples were dipped from the surface. If the phytoplankton was not plentiful, the samples were concentrated by pouring the water through a plankton net or by removing a portion of

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TABLE I

Dinoflagellates previously reported to be luminescent

Genus and species	Reference
Ceratium tripos	Michaelis, 1830; Reinke, 1898; Zacharias, 1905
Ceratium furca	Ludwig, 1898
Gymnodinium flavum	Kofoid and Swezy, 1921
Gymnodinium sanguineum	Hirasaka, 1922
Gonyaulax catenella	Sommer *et al.*, 1937
Gonyaulax monilata	Connell and Cross, 1950; Howell, 1953
Gonyaulax polyedra	Torrey, 1902; Kofoid, 1911; Haxo and Sweeney, 1955
Gonyaulax polygramma	Nishikawa, 1901
Noctiluca miliaris	Baker, 1753; Michaelis, 1830; de Quatrefages, 1850
Peridinium candelabrum	Ludwig, 1898
Peridinium eugrammum	Ludwig, 1898
Peridinium granni	Ganapati *et al.*, 1959
Peridinium seta	Ludwig, 1898
Prorocentrum micans	Ehrenberg, 1834
Pyrocystis noctiluca	Murray, 1885
Pyrodinium bahamense	Plate, 1906

the water through a filter while retaining the plankton.[4] Dinoflagellates were isolated from these samples during the day and were washed once or twice in filtered sea water. Ten to twenty identical cells were placed in each of four test tubes. The contents of one were fixed with Rodhe's iodine fixative and set aside for the identification of the genus and species. The other three tubes were placed in the window in natural light. Since it is known that the luminescence of some dinoflagellates is rhythmic, being much brighter at night (Sweeney and Hastings, 1957), all testing for luminescence was done during the evening following isolation. Two of the three tubes from the window were tested for light emission, with the photomultiplier photometer recording intensity. Stimulation of luminescence was provided by a stream of air (Sweeney, Haxo and Hastings, 1959). The contents of the fourth tube, as well as that of the two tubes from which recordings had been made, were examined for living cells. The fourth tube was included because the aeration may fragment the fragile dinoflagellates during testing. This examination was important in cases where no luminescence was recorded, to make certain that living material had been examined and thus avoid falsely negative reports. Examination just prior to testing was impossible since the jarring contingent on microscopic examination might have stimulated luminescence prematurely, and recovery of the power to luminesce is sometimes very slow.

To supplement the data obtained from plankton samples, cells from laboratory cultures were also examined by the same technique.

RESULTS

Most of the dinoflagellates common in the phytoplankton at La Jolla between October, 1956, and May, 1959, were tested for luminescence. Cells came directly from freshly collected water samples or were from cultures maintained in the laboratory. Some species were tested from both sources. The results of the study are presented in Table II. More than half of all the dinoflagellates tested proved to be non-luminescent. These included two species which had formerly been reported to emit light, *Prorocentrum micans* and *Ceratium furca.* Repeated tests of these species always gave negative results. All the species of *Gonyaulax* tested were luminescent. Some of these, like *G. polyedra* and *G. monilata,* were already known to be luminescent. Other genera represented contained both luminous and non-luminous species (*Ceratium* and *Peridinium*) or lacked luminous representatives (*Gymnodinium* and *Dinophysis*). In some cases, only a single species of a genus could be examined. It is interesting to note that while one culture of *Noctiluca* isolated from cells collected in the Gulf of California was

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[4] This method of concentrating phytoplankton samples, devised by Mrs. Anne Dodson of this laboratory, will be described in another publication.

TABLE II

Genus and species	Source*	Date tested
A. *Dinoflagellates found to be luminescent in this study*		
Ceratium fusus	S	10/18/56; 10/24/56; 11/8/56
Fragilidium heterolobum	S	3/20/57
Gonyaulax catenella	C	11/27/57
Gonyaulax hyalina	S	10/22/57
Gonyaulax monilata	C	11/27/57
Gonyaulax polyedra	C	1955–1963
Gonyaulax sphaeroidea	C	2/28/58
Noctiluca miliaris	C	1958–1959
Peridinium brochi	S	3/17/57
Peridinium conicum	S	3/10/58
Peridinium depressum	S	3/17/58
Peridinium pentagonum	S	5/7/57; 3/17/58
B. *Dinoflagellates found not to be luminescent in this study*		
Ceratium dens	S	10/24/56; 4/11/57; 4/29/57; 5/7/57; 5/21/57; 5/11/59
Ceratium furca	S	11/8/56; 3/13/57
Dinophysis fortii	S	3/24/58
Dinophysis caudata	S	3/7/57; 3/24/58; 4/7/58
Dinophysis tripos	S	3/7/57; 3/24/58
Diplopeltopsis minor	S	5/26/58
Gymnodinium splendens	C	11/27/57
Noctiluca miliaris (?)	S & C	5/3/57
Peridinium claudicans	S	3/17/58
Peridinium subsalsum	C	11/27/57
Peridinium trochoideum	C	11/27/57
Prorocentrum micans	S	10/24/56; 4/9/57; 5/21/57
	C	11/27/57
Scrippsiella sweeneyi	C	11/27/57
C. *Diatoms found not to be luminescent*		
Chaetoceros debilis	S	2/4/57
Coscinodiscus centralis var. pacifica	S	2/4/57
Ditylum brightwellii	S	2/4/57
Thallasiothrix mediterranea var. pacifica	S	2/4/57

* S designates cells isolated from the plankton at La Jolla; C, cells from cultures maintained in the laboratory.

brightly luminescent, two other cultures isolated from the La Jolla plankton were not luminescent. The cells of this *Noctiluca* were distinctly smaller even when they were growing rapidly in culture and the swarm spores were formed over an entire hemisphere of the cell rather than in a polar cap, as in the larger luminescent form, so that this may possibly be a different species (Eckert and Findlay, 1962).

Included for comparison are several diatoms from the phytoplankton. As expected, no diatom was found to emit light.

Indication of the reliability of the isolation and test methods was provided by the following observation. One sea water sample contained the following organisms in a 2-ml. aliquot:

Ceratium fusus	85
Ceratium furca	30
Prorocentrum micans	5
Chaetoceros (chains)	10
Ceratium sp.	2
Gyrodinium sp.	1
Silicoflagellate	1

The average luminescence emitted by such 2-ml. aliquots (10 samples) was 0.042 relative light units. Of the organisms found in this sea water, only *Ceratium fusus* tested positive for light emission, 20 cells yielding 0.0098 light units. This light emission is equivalent to 0.0416 light units from 85 cells. Thus, the light emitted by *Ceratium fusus* is sufficient to account for all the luminescence of the whole sample.

In general, then, many dinoflagellates belonging to a variety of general are able to emit light. However, by no means all species or even all genera possess this capacity.

The author wishes to express her gratitude to Dr. Enrique Balech and Dr. Robert W. Holmes for identifying the dinoflagellates in this study and to Mrs. Anne Dodson for technical assistance.

SUMMARY

The common dinoflagellates of the San Diego region have been isolated and tested for the ability to emit light. The results are presented in the form of a table, Table II. All the species of *Gonyaulax* which were tested were luminescent. Other genera in which more than a single species was examined included at least some non-luminous members.

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RESPIRATORY LEVEL OF SEA URCHIN EGGS BEFORE AND AFTER FERTILIZATION

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Following Warburg's experiments (1908), it was believed that the respiration of sea urchin eggs increased sharply at the time of fertilization. This phenomenon attracted much attention in the past and a number of papers appeared on the mechanism of respiratory increase at fertilization (*cf.* Runnström, 1949, and Rothschild, 1956, for references). Using the Cartesian diver technique, however, Borei (1948a, 1949) found that oxygen uptake of unfertilized eggs of *Psammechinus miliaris* decreased gradually following removal from the ovary. He assumed that there would be no increase in oxygen uptake if the eggs were fertilized immediately after shedding under natural conditions. According to his finding, the increase in oxygen uptake at fertilization seemed to be a special phenomenon and general interest in it diminished considerably. However, there were some data which threw doubt on the generalization of Borei's result. In the early study on *Arbacia* eggs, Tyler, Ricci and Horowitz (1938) showed that oxygen uptake of unfertilized eggs could remain fairly steady for several days starting shortly after removal of the eggs from the female. Cleland (1950) reported that in oyster eggs there was no gradual decline in oxygen uptake following removal from the ovary. On the other hand Tyler and Humason (1937) found that different batches of unfertilized eggs of the gephyrean worm, *Urechis caupo,* could have different absolute rates of respiration, depending upon their past history (storage time in the "nephridial sacs," etc.), some being higher than, some lower than, and some about the same as, that of the fertilized eggs. The latter showed about the same absolute rate regardless of batch. Because of the importance of Borei's finding, it was considered to be meaningful to repeat his experiments with different species of sea urchins. The present experiments showed somewhat different results from those of Borei. No decline in oxygen uptake of unfertilized eggs could be observed for some time after shedding. Further, the respiratory level of fertilized eggs was much higher than that of unfertilized eggs.

MATERIALS AND METHODS

Three species of Japanese sea urchins, *Hemicentrotus pulcherrimus, Pseudocentrotus depressus* and *Anthocidaris crassispina,* were collected at the Misaki Marine Biological Station and the Sugashima Marine Biological Station and used for experiments.

The eggs were obtained by injection of an isotonic KCl solution and were washed once with sea water. The oxygen uptake was measured both with the Cartesian diver respirometer and the Warburg manometer. The diver technique was essentially the same as that described by Holter (1943). The divers used in the present experiments were of standard type, having an air volume of 8–10 μl. They were charged in the usual manner. The Warburg flask constant was 0.33–0.65. Filtered sea water was used as the physiological medium throughout the experiments. As the measurements were taken at about room temperature, the diver and the flask reached their thermal equilibrium so quickly that it was possible to begin measurements in less than 20 minutes after shedding. The number of eggs was used as reference for the diver measurement, while total nitrogen was employed for the manometric method. To observe the respiratory change at fertilization, the divers were charged as described by Borei (1948b) and Nakano (1953). The unfertilized eggs were placed in the neck of the diver, and an alkali solution at the bottom. When a silicone-coated diver was used, the danger of premature fertilization was considerably reduced. While the diver was observed through the horizontal microscope, an overpressure was applied until eggs and sperm were mixed by the use of Claff's device (1949). This method permitted continuous measurement following fertilization. For the manometric method, the sperm placed in the side arm was added to the egg suspension in the main chamber after an appropriate time. After completing the measurements, the eggs were removed from the diver and the flask

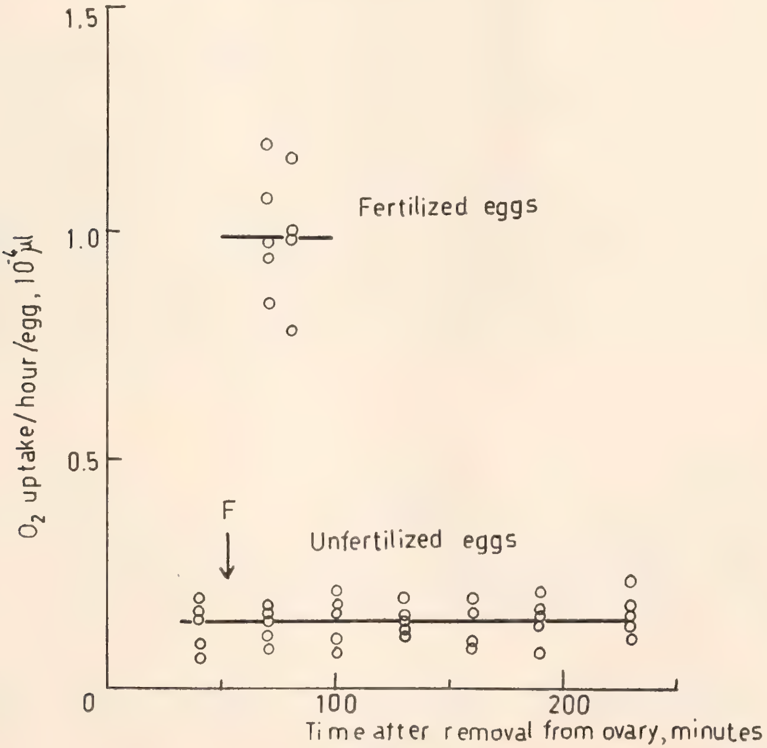

FIGURE 1. Oxygen uptake of eggs of *Pseudocentrotus depressus* before and after fertilization. F, addition of spermatozoa. Temperature, 18° C.

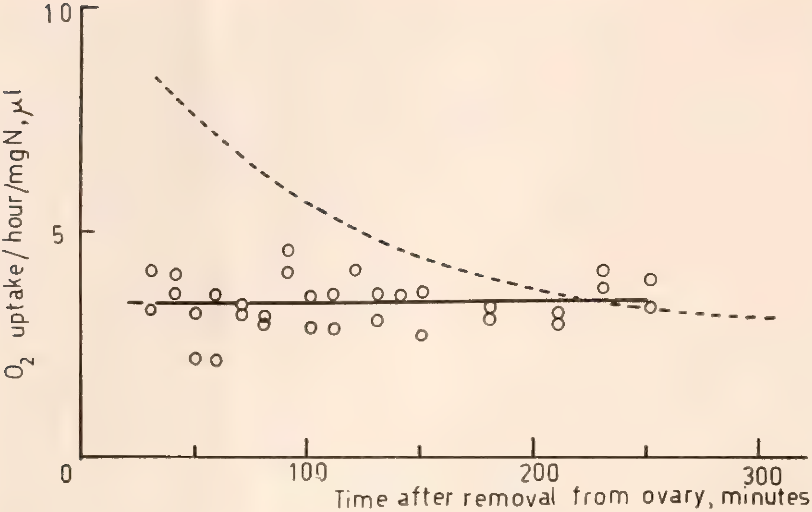

FIGURE 2. Oxygen uptake of unfertilized eggs of *Pseudocentrotus*. Temperature, 20° C. Dotted line is redrawn from Borei (1948a) for comparison.

and the percentage of fertilization was examined. Only fertilization above 90% was taken into consideration for results.

RESULTS

Figure 1 presents five diver technique experiments with *Pseudocentrotus* eggs. There is no gradual decline in oxygen uptake following removal from the ovary. A rather low rate of oxygen uptake is maintained for more than four hours from the time the first measurements were taken. The rate of oxygen uptake in fertilized eggs, however, is much higher than that of unfertilized eggs. Figure 2 shows two

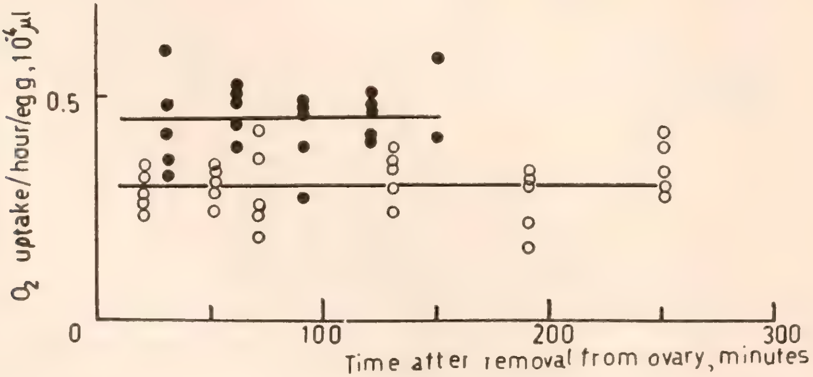

FIGURE 3. Oxygen uptake of unfertilized eggs of *Anthocidaris crassispina* (●) and *Hemicentrotus pulcherrimus* (○). Temperature, 30 and 18° C., respectively.

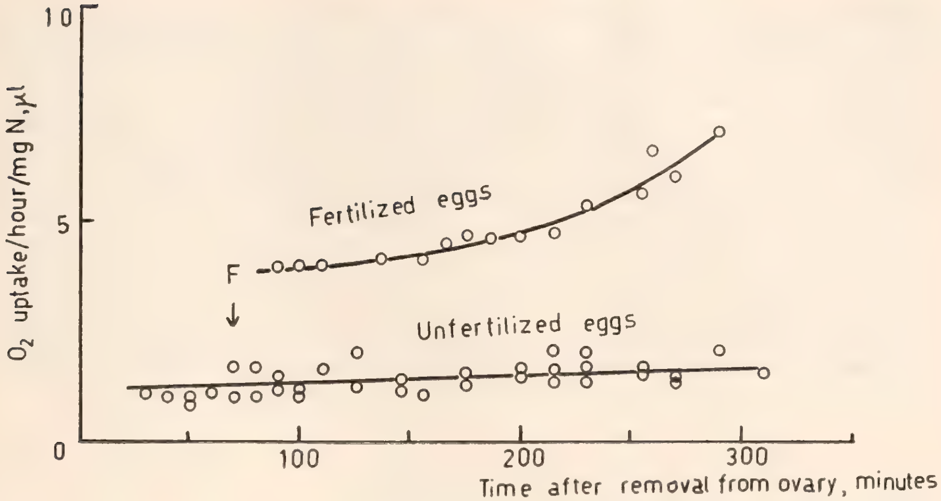

FIGURE 4. Oxygen uptake of *Hemicentrotus* eggs before and after fertilization. F, addition of spermatozoa. Temperature, 10° C.

manometric experiments with *Pseudocentrotus* eggs, neither one showing decline in oxygen uptake of unfertilized eggs.

The same results were obtained with *Hemicentrotus pulcherrimus* and *Anthocidaris crassispina.* It is clear from Figure 3 that oxygen uptake of unfertilized eggs immediately after shedding is low and remains so rather constant for three hours or more. When the eggs are fertilized, oxygen uptake increases sharply and the exponential increase starts as shown in Figure 4. These observations are in perfect agreement with those of previous reports of several investigators.

In Borei's experiment with *Psammechinus miliaris* (Z-form), the average rate of oxygen uptake in aged, unfertilized eggs and fertilized eggs is 0.47×10^{-4} and 1.84×10^{-4} µl./egg/hour, respectively. The values in the present experiment check well with those of Borei's experiment, except for the low rate in unfertilized eggs immediately after shedding.

DISCUSSION

The result of the present experiment seems to be in contradiction to Borei's finding (1948a, 1949). The respiratory level of unfertilized eggs turns out to be low even immediately after shedding and there is no gradual decline in oxygen uptake with time after shedding. In eggs of the species used, the sudden increase in oxygen uptake may occur at fertilization under natural conditions. These results seem to suggest that such a phenomenon may yet be important for the understanding of the fertilization mechanism, especially with regard to the activation of enzyme systems. Although there is a good deal of investigation about the mechanism of the sudden increase of oxygen uptake at fertilization in sea urchin eggs, nothing can be said with certainty. Runnström (1930) suggested that structural changes at the time of fertilization may induce contact between enzymes and substrates in respiratory chains of the egg. He also proposed a hypothesis that in unfertilized eggs an active cytochrome oxidase is present but only partly utilized (Runnström, 1956). In accord with this view, Maggio (1959) demonstrated the increase in the activity of cytochrome oxidase after fertilization. Other critical evidences that the cytochrome oxidase system was operative in unfertilized sea urchin eggs were obtained by Robbie (1946) and Black and Tyler (1959a, 1959b). Recently, Gonse (1960) compared the respiratory level of unfertilized eggs with that of fertilized eggs and suggested that no major change in the enzyme system is involved at the time of fertilization. As to the low respiratory level in unfertilized eggs, there is another possibility that they may contain some inhibitors of cellular oxidation (Runnström, 1949). In fact, Maggio and Monroy (1959) showed an inhibitor of cytochrome oxidase in the supernatant of egg homogenates, whose nature is still obscure. At present it may be too early to draw any conclusion concerning the mechanism of respiratory change occurring at fertilization, but the present result may justify further efforts toward its elucidation.

The authors wish to acknowledge with gratitude the cooperation of Mr. Hideo Tomita, of Nagoya University, in the experiments.

SUMMARY

The respiratory level of sea urchin eggs was measured before and after fertilization, using three Japanese species, *Pseudocentrotus depressus*, *Hemicentrotus pulcherrimus* and *Anthocidaris crassispina*. The rate of oxygen uptake of unfertilized eggs is low even immediately after shedding, and it remains constant for more than three hours. Upon fertilization, the rate of oxygen uptake of the eggs increases sharply, and the increase may occur under natural conditions.

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EFFECT OF SALINITY ON GROWTH OF POSTLARVAL PENAEID SHRIMP[1]

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The general life-history pattern of commercially important penaeid shrimps of the northern Gulf of Mexico has been known for some years (Pearson, 1939; Weymouth, Lindner and Anderson, 1933; Burkenroad, 1939). That the adults spawn offshore, the young migrate shoreward to the estuaries for a period of rapid growth, and then return to the offshore areas is generally accepted. However, neither the stimulus for the migrations nor the factors controlling the period of time spent in the estuaries are well understood. Several explanations for this behavior have been advanced. Some authors indicate that salinity *per se* is of prime importance. For example, Pearse and Gunter (1957) state (p. 147): "The young of many animals, usually thought of as marine, require areas of low salinity for nursery grounds. The distribution and abundance of the blue crab and the commercial shrimp (*Penaeus setiferus*) on the South Atlantic and Gulf coasts are dependent on the presence of estuarine areas. The shrimp spawn in oceanic salinities; the early stages apparently require oceanic water, but the older larvae must reach bay waters or perish. The young shrimp grow up in the low-salinity bays and return to the sea." Other authors, including Lindner and Anderson (1956) and Hoese (1960), have felt salinity to be of less importance. Broad (1962, p. 1), referring to penaeids, remarks: "Thus, while almost every investigator during the last thirty years has considered salinity in relation to shrimp distribution . . . it is still not possible to state what effect salinity may have, for example, on the growth of shrimps." The following experiments were designed to evaluate the effects of salinity upon the growth of postlarvae of the white shrimp, *Penaeus setiferus*, and either the brown shrimp, *P. aztecus*, or the pink shrimp, *P. duorarum.*

MATERIALS AND METHODS

Two series of growth experiments were conducted, the methods being generally the same in each. Large numbers of postlarval shrimp were seined from nearby surf zones for use as experimental material in both series. Prior to transfer into experimental vessels, the small shrimp were held for 24 hours under laboratory conditions in quantities of the same water in which they were caught, or in water of the same salinity. One hundred postlarval penaeids were placed in each experimental tank; the salinity of the medium at this time was identical to that at which the animals had been caught. Salinity was decreased stepwise by removing sea water and replacing it with an equal volume of distilled water. Salinity was increased similarly using concentrated sea water in place of distilled water. Stepwise salinity changes required 48 hours in the first series (GS-1) and 120 hours in the second (GS-2) (Table I).

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The experimental vessels consisted of 20-gallon glass aquaria fitted with "Eureka" inside plastic filters which filtered and aerated the water. The filters were covered with ground oyster shell topped with fine beach sand, the combination serving both as a filter bed and as a substrate for the animals. Both shell and sand were washed and heat-sterilized before use. At the start, 40 l. of sea water were added to each aquarium. The initial salinity of the water was 25‰ (parts per thousand) in series GS-1 and 39‰ in series GS-2. Salinity was determined semi-weekly using a hydrometer.

A thin (2-mil) polyethylene film was placed in contact with the surface of the water in the experimental tanks to reduce evaporation and to prevent escape of the postlarvae. During preliminary work in the absence of the film, as many as 20 postlarvae had been found trapped on the side of a tank in a single day. That this method was successful in reducing such losses is indicated by the 100% survival noted in some sections of the experimental series.

Table I

Schedule of salinity changes

Date	Elapsed time (hr.)	Initial salinity (‰)	Desired salinity				
			2‰	5‰	10‰	25‰	40‰
Series GS-1:							
4/12/62	2	25	—	20	20	No change	33
	7		—	15	15	No change	38
4/13/62	26		—	10	10	No change	40
	31		—	5	10	No change	40
Series GS-2:							
7/26/62	3	39	35	35	35	35	40
	8		30	30	30	30	40
7/27/62	24		25	25	25	25	40
	31		21	21	21		
7/28/62	48		17	17	17		
	55		13	13	13		
7/29/62	72		10	10	10		
	63		7.5	7.5			
7/30/62	96		5.0	5.0			
	104		2.0				

The schedule of salinity changes in the two series is given in Table I. Salinity reduction required more time during the second series because of the higher initial salinity. In neither series were deaths noted during the acclimation period.

During both series of experiments the laboratory temperature was maintained at 23 to 25° C. while the water temperature ranged from 24.5 to 26° C. Each aquarium was continuously illuminated by two 14-watt daylight fluorescent bulbs.

It should be noted that the animals in the two series were caught at different water temperatures. Animals in series GS-1 came from water of 23° C. whereas those in GS-2 came from water of 30° C.

Initial measurements in each experiment were obtained from samples of 10 postlarval shrimp withdrawn from the source population. After excess water was removed, the length of each specimen was measured to the nearest 0.5 mm. (distance from tip of rostrum to end of telson), and its weight determined to the nearest 0.1 mg. on a Mettler H15 analytical balance. All individuals were preserved for later study. Subsequent samples from each experimental tank were similarly treated after being withdrawn at approximately 5-day intervals. Both the largest and the smallest specimens in each tank at each sampling period were included in order to determine size ranges; the remaining eight were selected at random. After approximately 30 days, the surviving animals in each tank were weighed individually and preserved.

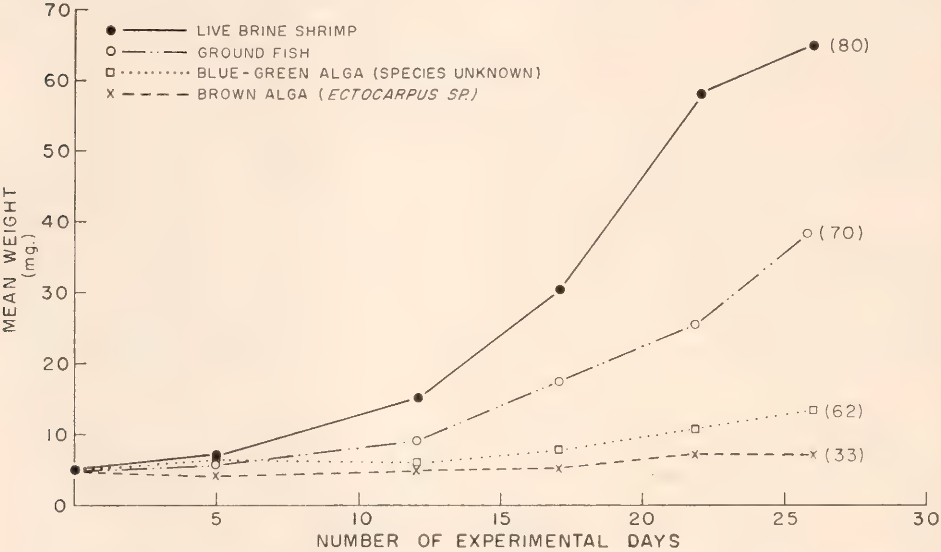

FIGURE 1. Growth of postlarval penaeids fed various diets. Figures in parentheses indicate percent survival at termination of experiment.

Preliminary experiments which were of similar design and carried out at 25‰ had indicated that a live food was to be preferred. Figure 1 not only indicates that growth was substantially better among the postlarvae fed brine shrimp, *Artemia*, nauplii, but that survival was also greater. As Williams (1959) has previously noted in the brown shrimp, those animals fed algae always had full guts and deposited deep green or brown feces, but neither their growth nor their survival approached the levels attained by animals fed the brine shrimp diet. In addition to a slightly poorer growth rate among animals fed ground fish or shrimp, there was a practical problem caused by fouling of the water with excess food. For these reasons, brine shrimp nauplii constituted the sole dietary supply in the salinity experiments described. Laboratory cultures of brine shrimp were filtered and washed before use in order to avoid salinity changes in the experimental tanks caused by the addition of saline (brine shrimp) culture medium. Equal amounts of brine shrimp were given to postlarvae in all tanks. The volume and number of feedings per day were increased during the experiment in order to maintain an excess of food in the tanks.

Although all the shrimp in series GS-1 (April, 1962) were tentatively identified as grooved shrimp, *i.e.*, brown shrimp or pink shrimp, those obtained for GS-2 (July, 1962) apparently included both "grooved" and white shrimp in an approximate ratio of five grooved to one white. Tentative species identification was made following Williams (1959), with early postlarvae (less than 10 mm. rostrum-telson length) being differentiated on the basis of ratio of distance from rostrum to eye and distance from third pereiopod to eye, and specimens longer than 17 mm. on the basis of the presence or absence of a groove. Conflicting evidence is available concerning identification at lengths between 10 and 17 mm. Thus, identification must be regarded as tentative, although some confirmation of species ratio was available from the animals surviving for 30 days.

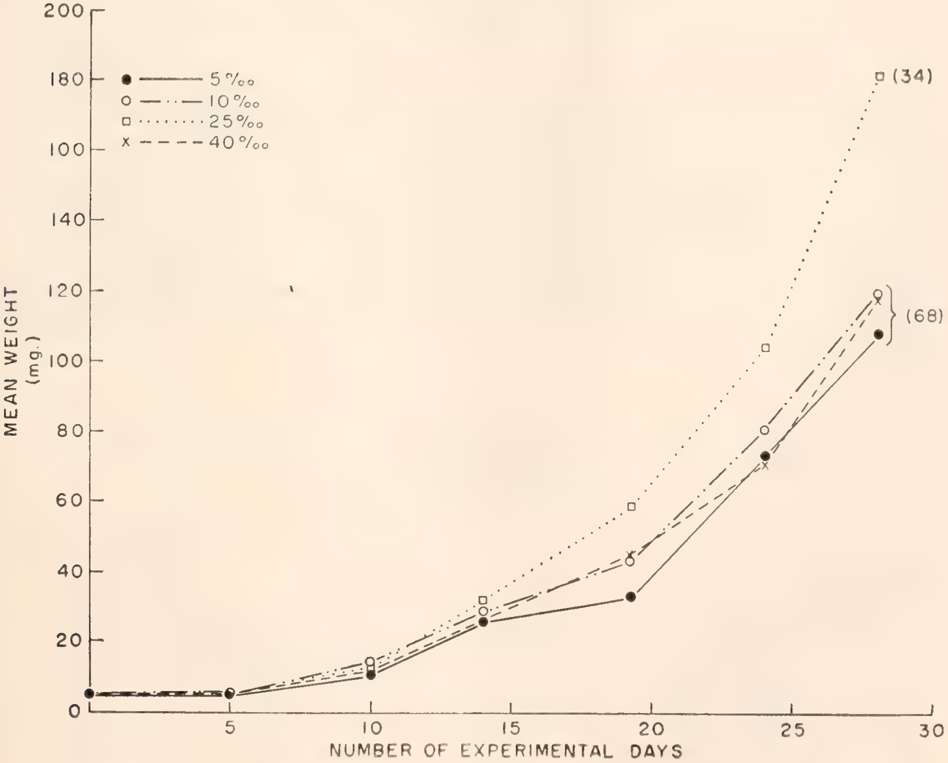

FIGURE 2. Growth of postlarval penaeids at various salinity levels in series GS-1. Figures in parentheses indicate percent survival at termination of experiment.

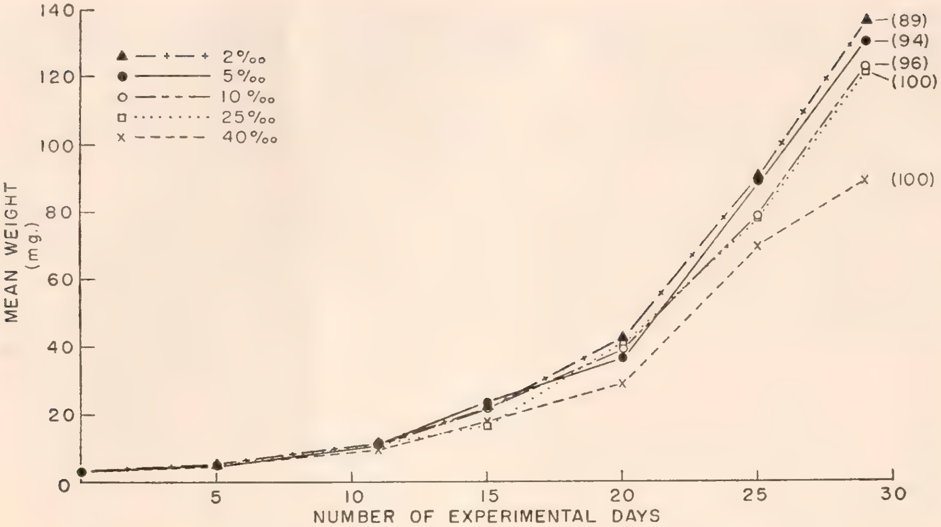

FIGURE 3. Growth of postlarval penaeids at various salinity levels in series GS-2. Figures in parentheses indicate percent survival at termination of experiment.

As indicated in Figures 2 and 3, growth of the postlarvae occurred at all salinity levels tested in both series of experiments. Growth has been expressed as total weight in mg. (Tables II and III) rather than as total length since, as shown in Figure 4 (which is based on animals measured during these experiments), weight increases more rapidly than length and is therefore less influenced by relative measurement error. Thus, in the first 5 days, weight increased by about 50% while total length increased only 15%. In each subsequent 5-day period weight generally doubled while length was increasing only 25%. For this reason weight has been used here as a more sensitive indicator of growth than total length.

TABLE II

Mean animal weight (in mg.) at each salinity level in experimental series GS-1. Values are based on weights of 10 animals. Figures in parentheses indicate one standard error

Elapsed days	Salinity level			
	5‰	10‰	25‰	40‰
0	—	—	4.5 (0.2)	—
5	5.7 (0.3)	5.4 (0.6)	6.6 (0.4)	6.2 (0.4)
10	14.1 (1.4)	10.9 (0.9)	13.0 (1.5)	11.9 (0.8)
14	29.1 (3.3)	26.2 (3.6)	32.6 (4.4)	26.3 (4.2)
19	43.8 (5.0)	32.7 (6.0)	58.9 (10.6)	45.4 (10.4)
24	81.4 (9.2)	72.9 (8.6)	104.7 (14.2)	71.3 (6.8)
28	119.6 (13.6)	108.7 (18.6)	182.0 (18.8)	118.0 (19.1)

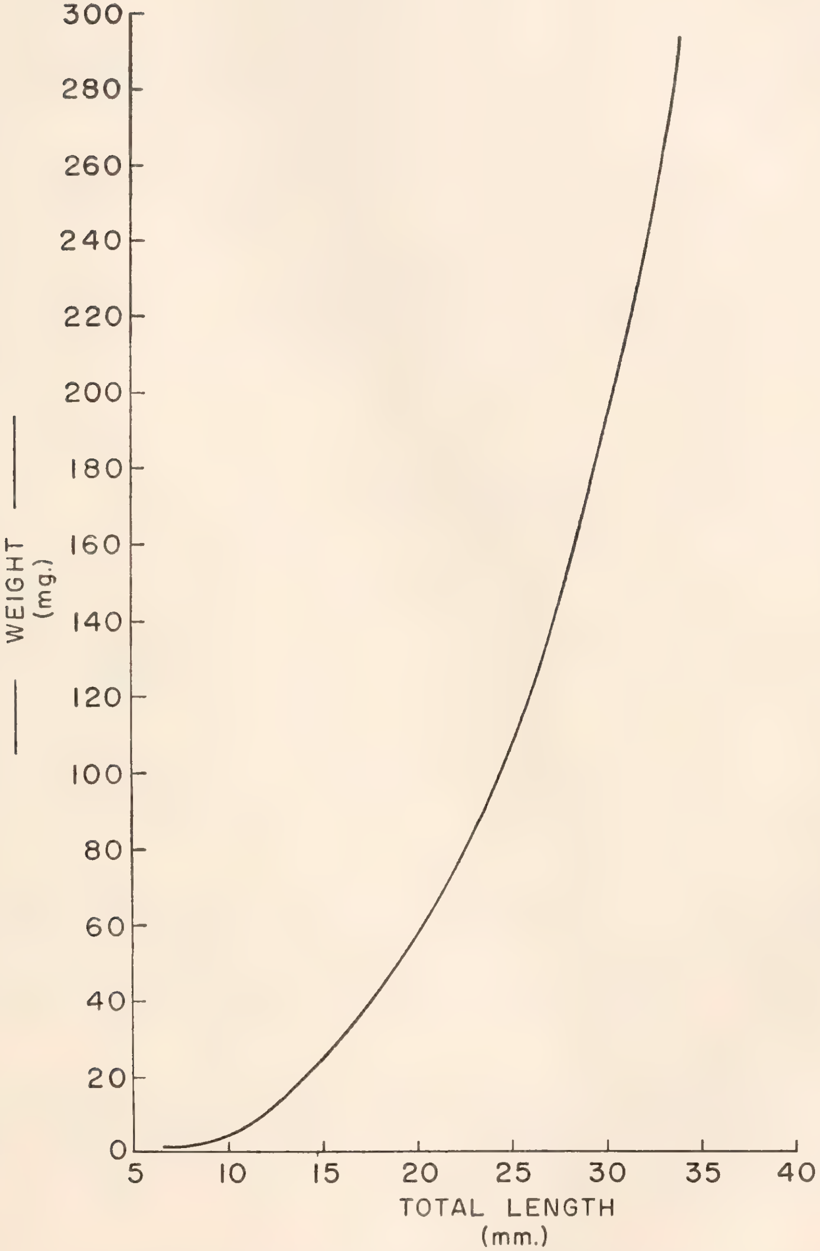

FIGURE 4. Weight-length relationship in postlarval penaeids.

Although growth appeared to be much greater in the first series at the 25‰ salinity level, it must be noted that the per cent survival was considerably less than that at the other salinities (Fig. 2). Comparison by *t* tests of mean weight increments at 25‰ *vs.* those at other salinity levels revealed that growth at the former level was greater throughout the series than at any other level tested. The increased growth rate may be related to the fact that more food was available for each surviving animal as a result of the increased mortality, or it may be a simple reflection of the decreased population density. There was no apparent reason for the greater mortality in this tank, and, indeed, as noted in earlier feeding experiments (all conducted at 25‰) as well as in the later salinity series GS-2, both growth and survival were rated as excellent at this salinity. No significant difference between other pairs of means in this series could be detected.

In the second series of experiments (GS-2), survival was considerably better than that occurring during the first series. All test animals survived at both 40‰ and at 25‰, and survival in no case was less than 84%. This better survival may have been due to a difference in the medium (sea water for GS-2 was obtained directly from the surf zone whereas that for GS-1 came from a large recirculating sea-water system), or to better feeding. It is significant that 100% survival occurred in the two highest salinity levels and that considerable growth occurred even at 40‰. This would indicate that postlarval penaeids are not only able to withstand such high salinity for a fairly long period of time, but can grow at a rate nearly as great as that attained at presumably more "normal" levels.

TABLE III

Mean animal weight (in mg.) at each salinity level in experimental series GS-2. Values are based on weights of 10 animals. Figures in parentheses indicate one standard error

Elapsed days	Salinity level									
	2‰		5‰		10‰		25‰		40‰	
0	—		—		—		2.9	(0.2)	—	
5	4.8	(0.2)	4.8	(0.2)	4.3	(0.1)	4.3	(0.4)	4.3	(0.4)
11	11.1	(1.6)	10.8	(1.2)	11.0	(1.0)	9.9	(1.2)	9.9	(1.2)
15	21.2	(2.7)	23.1	(4.0)	22.6	(3.5)	16.5	(2.0)	17.8	(2.9)
20	42.6	(7.3)	36.5	(9.0)	36.9	(6.3)	40.3	(7.1)	19.1	(2.3)
25	90.2	(21.2)	88.9	(24.4)	78.6	(17.0)	77.3	(12.2)	69.6	(10.6)
29	140.9	(23.3)	147.4	(27.7)	135.0	(31.2)	138.4	(22.2)	94.7	(10.4)

It is interesting to note that the rate of growth varied considerably among individuals within the same tank, regardless of the salinity. Specimens in initial samples ranged from 3.6 to 5.6 mg. and from 10.0 to 11.0 mm. in GS-1, whereas animals in GS-2 ranged from 1.3 to 3.8 mg. and from 6.5 to 10.0 mm. However, at 29 days, when experiment GS-2 (25‰) terminated, weights varied between 39.5 and 305.4 mg. and lengths between 17.0 and 36.5 mm. Such variation was noted during both series of experiments. In each, growth exceeded that recorded by Pearson (1939) for either white or "brown" shrimp postlarvae in laboratory aquaria, but was probably less than that occurring under natural conditions.

Discussion

Although salinity has long been considered a major factor in the ecology of commercial penaeid shrimps, few data have been available either to substantiate or negate claims that low salinity is a requirement for the growth of postlarval shrimp. That postlarval shrimp are found in low salinity water is an undisputed fact, but the influence of salinity *per se* on this distribution has not been determined. Lindner and Anderson (1956) reported that the size of young shrimp was correlated more with locality than salinity and concluded that salinity within broad ranges was not important. Hoese (1960) states (p. 593): "It would seem that within certain areas, at least, salinity in broad ranges is inconsequential to young shrimp. All the habitats where juveniles have been found could offer protection from predators. . . . It appears that juvenile *P. setiferus* and *P. aztecus* can populate areas of relatively high salinity if other environmental factors are ideal." Gunter, too, has modified his stand concerning the importance of salinity. He states (1961, p. 599): "Small shrimp are not killed or precluded by high salinity as if it were poison; they simply do not do well in it, for reasons unknown." Nevertheless, he mentions finding 115 white shrimp ranging in length from 15–66 mm. in the Laguna Madre at a salinity of 41.3‰. Johnson and Fielding (1956), in attempts to rear shrimp in ponds, noted that white shrimp could be raised at salinity levels of 18.5 and 34‰, and Williams (1960), in experiments concerning the osmotic relationships of brown and pink shrimp, found that temperature apparently had far more effect upon survival than did salinity. The experiments presented here indicate that under the conditions tested (restricted temperature and diet), postlarval shrimp can both survive and grow over a wide range of salinities. Thus, it would appear that salinity tolerances *per se* may not play a direct role in the growth and survival of postlarval and juvenile shrimp in the estuarine environment.

It may be tentatively suggested that food requirements may be of more importance than the purely physical factors. Some indication of this food requirement was noted during these experiments, for it was, in fact, the inability to provide food in sufficient quantity which terminated the experiments after only a month. The relationship of growth to utilization of food is now being studied in an effort to obtain an estimate of the food required to produce a given weight of shrimp. Further studies will test the effect of population density upon growth. Future work will also include experiments concerning the interrelation of temperature and salinity and their combined effect upon the growth of postlarval shrimp.

The method of preventing postlarval escape by use of polyethylene film was designed by Dr. David V. Aldrich. His help and the assistance of Mr. Don S. Godwin and Mr. Roger M. Friedberg is gratefully acknowledged.

Summary

1. The effect of salinity on the growth and survival of postlarvae of white, *Penaeus setiferus*, and grooved shrimp, *P. aztecus* or *P. duorarum*, has been studied in the laboratory.

2. Growth rate did not differ significantly among shrimp held at 2, 5, 10, 25, or 40‰.
3. Survival was generally excellent at all salinity levels tested, including 40‰.
4. The results suggest that salinity *per se* does not limit growth of young shrimp.

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STIMULATION OF SPAWNING IN THE MUSSELS, MYTILUS EDULIS LINNAEUS AND MYTILUS CALIFORNIANUS CONRAD, BY KRAFT MILL EFFLUENT[1]

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Various procedures have been employed by different investigators to stimulate spawning in the bay mussel, *Mytilus edulis* Linnaeus, and the California sea mussel, *M. californianus* Conrad, in the laboratory. These methods have met with varied success in terms of ease, efficiency, and reproducibility. Field (1922) claimed that he was able to induce spawning in the bay mussel within an hour by rough handling, such as shaking the animals in a bucket. Costello *et al.* (1957), however, were unable to stimulate spawning in the bay mussel by this treatment. The latter authors were able to obtain gametes, if the gonads were fully ripe, by removing the valves of an animal and placing the mantle folds in a small bowl containing about 100 cc. of sea water. Just (1939) was able to obtain gametes from the bay mussel by placing intact animals in bowls with sea water and allowing natural spawning to occur. Similarly, Berg and Kutsky (1951) obtained gametes from the bay mussel by allowing natural spawning to occur in bowls of sea water following storage of the animals in a dry state in a refrigerator at 4° C. Under these conditions spawning occurred up to one week after storage. However, it was not clear from these reports how readily spawning occurred, or how efficient was the method.

Iwata (1950a, 1950b; 1951a, 1951b, 1951c, 1951d, 1951e, 1951f, 1951g; 1952) studied the stimulation of spawning in *M. edulis* under more precisely controlled conditions. He found that spawning occurred in intact animals within 30–60 minutes after an electrical stimulation of 20 volts for 5 seconds, or within 1–5 hours after an injection of 0.5 cc. of $M/2$ KCl, but not sea water, into the mantle cavity. Spawning also occurred within 30–90 minutes if mantle pieces were bathed for 5–10 minutes in $M/2$ KCl, $M/2$ NH_4Cl, or $M/3$ $BaCl_2$; stimulated electrically; or sub-

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jected to a sudden temperature rise from 7° to 15° C; $M/2$ NaCl, $M/2$ LiCl, $M/3$ $SrCl_2$, $M/3$ $CaCl_2$, and $M/3$ $MgCl_2$ did not induce spawning from mantle pieces. Ova obtained under these conditions were mature and were fertilized by sperm obtained in the same manner.

Sugiura (1962) has also shown that excised mantle pieces from hermaphroditic *M. edulis* can be induced to spawn by electrical stimulation.

In the case of the sea mussel, Young (1945) reported that very few animals would spawn spontaneously in the laboratory either as a result of exposure to air or by transfer from one container to another. Likewise, a gradual or sudden rise in temperature was ineffective in inducing spawning. However, mechanical stimulation, such as scraping the shells to remove adherent organisms or pulling the byssus, or sexual stimulation were very effective in inducing spawning within 1–6 hours following stimulation. The latter method involved the placing of spawning mussels into containers with the ones to be tested, or introducing freshly deposited spawn or macerated gonadal tissue into the containers holding the test animals. Very few control animals, maintained in sea water in bowls, spawned after 10 hours, but the majority of the experimental animals did so following stimulation by either of the above methods.

In view of the variability encountered with the various methods used for the stimulation of spawning in mussels, it was deemed advisable to present in detail the results of experiments performed monthly during 1959–1963 on the stimulation of spawning in the bay mussel by kraft mill effluent (sulfate process pulp mill waste). The experiments on the sea mussel were carried out only on animals collected during January and February, 1963. The present report is concerned with these findings.

MATERIALS AND METHODS

Bay mussels were collected from pilings in Yaquina Bay, and Alsea Bay, Oregon, and sea mussels from rocks from a protected outer coast at Seal Rock, and at Otter Rock, Oregon. The animals were scraped free of adherent organisms on the day of collection, maintained in a dry state overnight at room temperature, and used the following day.

Experiments on intact animals were carried out in small fingerbowls, with one mussel per bowl. Control mussels were maintained in 200 cc. of fresh sea water, and the test mussels in 200 cc. of 4% kraft mill effluent (KME), diluted by volume with sea water, obtained from a local paper mill. In some experiments, the stimulatory activity of KME was also tested by removing one-half of the control animals from the sea water after 4–5 hours and placing them in KME.

Animals were subjected to an electrical stimulation of 16 volts alternating current for 15 seconds by carefully separating the valves sufficiently to permit the insertion and placement of the electrodes in contact with the mantle. Following stimulation the mussels were maintained individually in 200 cc. of sea water in finger bowls. Controls consisted of unstimulated and sham operated animals. Four hours after stimulation, mussels in all three groups which had not spawned were tested for reactivity to KME by placing them in 200 cc. of a 4% dilution of this material.

All experiments were conducted at approximately 20° C. Following stimulation the animals were observed for 24 hours for the emission of gametes.

To test for maturity of ova and viability of sperm following KME stimulation, several mussels in each experiment were removed from the KME solution at the first sign of spawning, washed, and placed in clean sea water. Under these conditions the animals continued to spawn and the gametes so obtained were harvested and used in fertilization tests.

RESULTS

The results of experiments on the stimulation of spawning in mussels by KME are summarized in Table I. It is clear that KME has a marked stimulatory activity on mussels, whereas very few of the control animals, maintained in sea water, spawned during the 24-hour observation period. Further evidence in support of the stimulatory activity of KME is seen in experiments 10, 11, and 12, where mussels were placed in KME after 5 hours in sea water. Under these conditions no mussels spawned before but many did so after stimulation. The reaction time, the time between the application of the stimulus and the first appearance of gametes, in these experiments varied between 20 minutes and 24 hours, with 41% of the animals spawning within the first hour. There was no sexual difference in responsiveness to KME. Approximately equal numbers of males and females spawned in each experiment. The gametes obtained by KME stimulation were

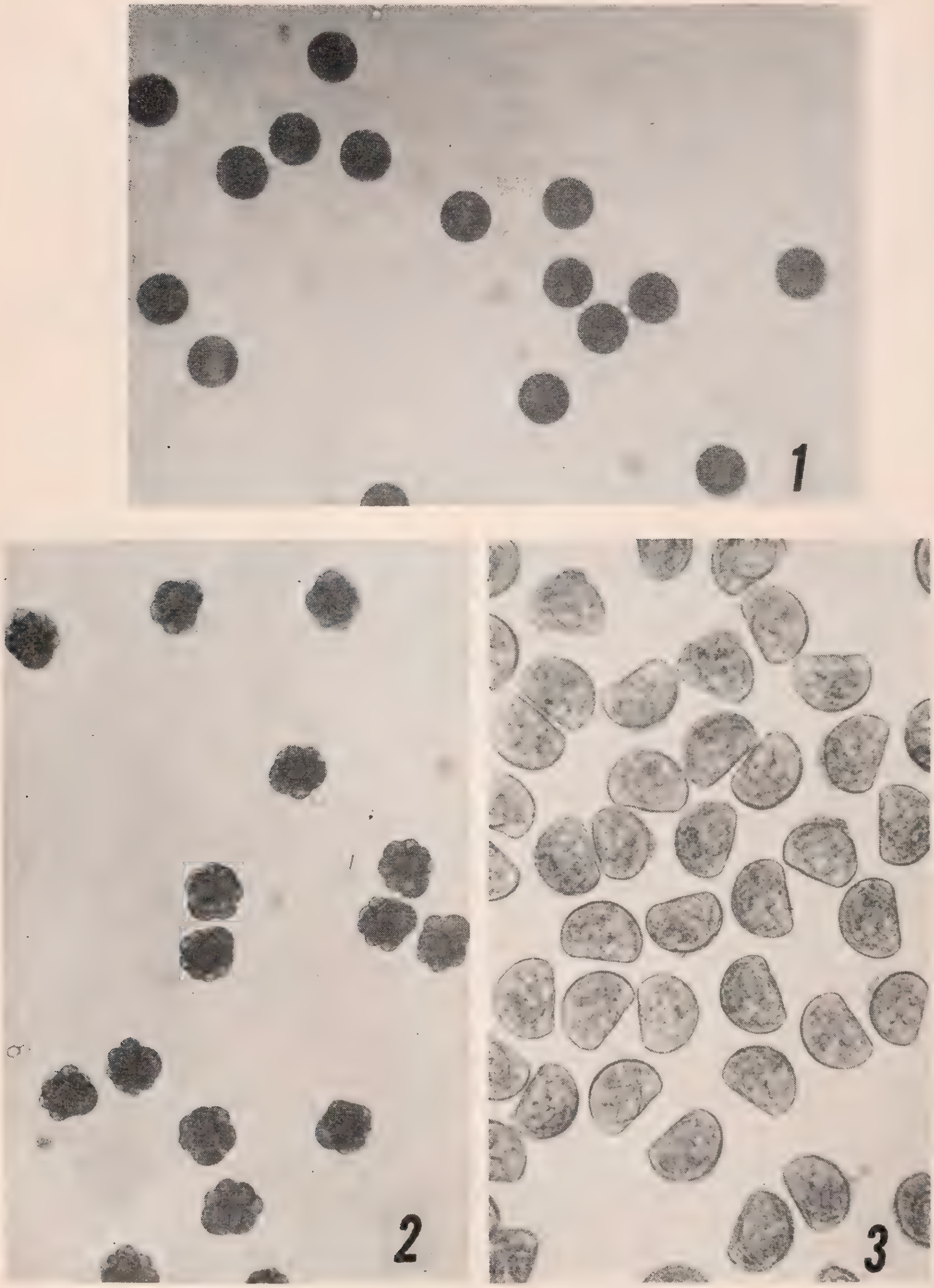
TABLE I

Effect of kraft mill effluent (KME) on spawning in Mytilus edulis *and* M. californianus

Exp. No.	KME*		Control		Control + KME**		
	No. spawned No. tested	% Spawned	No. spawned/ No. tested	% Spawned	No. spawned/ No. tested	% Spawned Before KME	% Spawned After KME
M. edulis							
1	19/29	66	2/30	7			
2	6/30	20	1/30	3			
3	17/30	57	1/30	3			
4	17/30	57	0/30	0			
5	18/30	60	2/30	7			
6	19/30	63	1/30	3			
7	31/90	34	0/30	0			
8	24/30	80	0/18	0			
9	50/60	83	1/28	4			
10	4/30	13	0/30	0	7/30	0	23
11	10/30	33	1/30	3	11/30	0	37
M. californianus	2/30	7	0/30	0	4/30	0	13

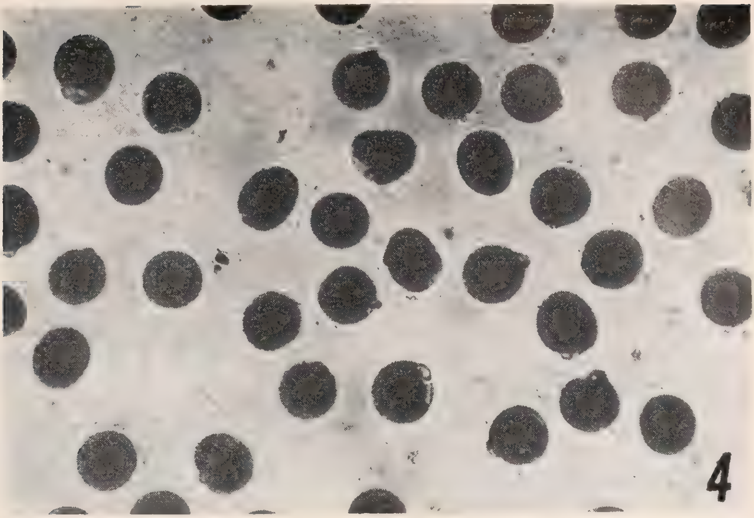
* 4% KME in 200 cc. of sea water.

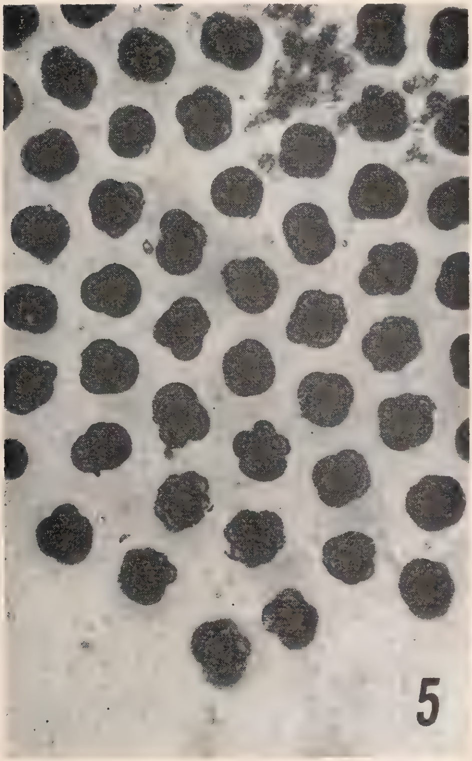
** Animals placed in KME after 5 hours in sea water.

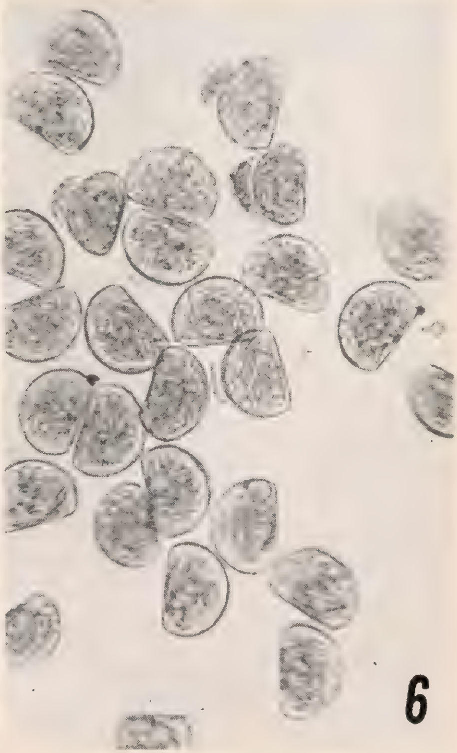


Development of *M. edulis* following artificial fertilization of gametes obtained from mussels stimulated with KME. × 100.

FIGURE 1. First polar body, 40 minutes.
FIGURE 2. Eight-sixteen-cell stage, 200 minutes.
FIGURE 3. Shell stage, 48 hours.







Development of *M. californianus* following artificial fertilization of gametes obtained from mussels stimulated with KME. × 100.

FIGURE 4. First polar body, 75 minutes.
FIGURE 5. Four-eight-cell stage, 165 minutes.
FIGURE 6. Shell stage, 48 hours.

viable and capable of fertilization (Figs. 1–6). The approximate times, for both species, for the appearance of the first polar body, 2–4-cell stage, swimming stage, and shell stage at room temperature were 40–75 minutes, 90 minutes, 18 hours, and 48 hours, respectively, after fertilization.

In Table II are summarized the results of experiments on spawning of mussels by electrical stimulation, as well as by KME stimulation. The evidence suggests that electrical stimulation *per se* may be effective in triggering spawning, although some of the sham operated controls also spawned. Of interest was the finding that animals which did not respond to electrical stimulation or to the sham operation were capable of subsequent stimulation by KME, although more of the animals in the latter group did so.

TABLE II

Effect of kraft mill effluent (KME) and electrical stimulation on spawning in Mytilus edulis *and* M. californianus

KME		Electricity		Sham Controls*		Controls	
No. spawned/No. tested	% Spawned	No. spawned/ No. tested	% Spawned	No. spawned/ No. tested	% Spawned	No. spawned/ No. tested	% Spawned
M. edulis 15/25	60	9/25	36	4/25	16	2/25	8
		After KME Stimulation**					
		5/16	31	14/21	67	8/23	35
M. californianus 8/25	32	4/25	16	2/25	8	0/25	0
		After KME Stimulation					
		0/21	0	2/23	9	3/25	12

* Not stimulated electrically but otherwise treated in an identical manner.
** Non-spawning animals exposed to 4% KME after 4 hours.

DISCUSSION

The results of the experiments reported here demonstrate that KME is a highly effective stimulus in triggering spawning in the bay and California sea mussels. Moreover, this material will stimulate spawning in bay mussels obtained during all times of the year, although to a somewhat lesser extent in animals collected during the months of October through December. This has been repeatedly confirmed in this laboratory in many other tests in which KME was used to trigger spawning in bay mussels in order to obtain gametes for use in bio-assay tests on the toxicity of various concentrations of mill effluents to developing mussel embryos.

Thus, the technique described here to induce spawning is simple, efficient, and affords a high degree of reproducibility. In addition, stimulation of spawning by KME does not affect the viability and fertilization capacity of the gametes, since the ova and sperm so obtained are fully capable of fertilization with development of the embryos to the shell stage.

The results reported here on the stimulation of spawning in mussels by KME complement the observations of others that certain chemicals are very effective in this regard. Iwata (1951f) has demonstrated that solutions of KCl, NH_4Cl, and $BaCl_2$ stimulated spawning in mantle pieces of *M. edulis*. We have also found that intact bay mussels will spawn when placed in 0.03 *M* KCl solution in sea water (unpublished observations). Sagara (1958) has shown that other bivalves can be stimulated to spawn by immersion in ammoniated sea water or by injection of NH_4OH into the gonads.

We have not been able to confirm the observations of Field (1922) and Young (1945) that rough handling, as scraping the shells, or changing the water (Just, 1939) will consistently induce spawning to any great extent. Costello *et al.* (1957) also were unable to stimulate spawning of the bay mussel by rough handling. Mild stimulation of this sort may induce spawning in a few individuals and may be the reason for the spawning in some of our control animals. It appears, however, that more severe trauma, such as partial opening of the shell, as reported here, or complete removal of the valves (Costello *et al.,* 1957), is necessary to induce spawning in a large number of animals.

Our results agree with those of Iwata (1950b) showing that electrical stimulation of intact mussels will trigger spawning; however, this stimulus is not as effective as KME.

The basis for the mechanism of spawning in mussels is not clearly understood. Iwata (1952) has shown that spawning in *M. edulis* can be induced by bathing mantle pieces in solutions of KCl, NH_4Cl, $BaCl_2$ and NH_4OH, but not in NaCl, LiCl, $SrCl_2$, $CaCl_2$ and $MgCl_2$ solutions. He explained these results on the basis of the higher mobility of the cations in the former group, and concluded that these chemical stimuli in the female do not act directly on the eggs, but rather upon the ovarian cells. The excitation of these cells then results in the maturation and subsequent release of the eggs. Lubet (1956) believes that spawning in *M. edulis* is dependent on the interaction between external and internal factors. He found that ablation of the cerebroid ganglia resulted in an accelerated spawning and concluded that the neurosecretion of these organs exerted an inhibitory action. Removal of this inhibition would then permit the animal to become receptive to the external stimuli resulting in the emission of gametes. This relationship may explain the refractoriness of certain animals subjected in our experiments, and in the experiments of others, to the external stimuli. Such stimuli then would not always act with equal efficiency on all animals.

The exact nature of the material in KME responsible for the triggering of spawning is not known. KME (combined wastes from sulfate process pulp mills) is a highly complex material and is a partial result of the alkaline digestion of wood chips. Isolation and characterization of the stimulatory material, if possible, may aid in elucidating the mechanism of spawning in mussels. Experiments bearing on this point are in progress.

It must be emphasized that stimulation of spawning in mussels by KME is a laboratory phenomenon and there is no definitive evidence that this material acts in a similar manner under natural conditions. It is unlikely that concentrations of KME in marine receiving waters would be sufficiently high to trigger spawning of mussels in their natural habitats except perhaps in the immediate vicinity of waste outfall.

SUMMARY

1. Kraft mill effluent is a very effective material in stimulating spawning in the mussels *M. edulis* and *M. californianus*. Bay mussels obtained at all times of the year and sea mussels during January and February from the Oregon coast spawned within 24 hours after exposure to 4% KME. The gametes so obtained were viable and capable of fertilization, as shown by the artificial fertilization and development of eggs to the shell stage.

2. Electrical stimulation of intact mussels was also shown to stimulate spawning, but was not as effective as KME.

3. Mechanical stimulation, such as scraping the shells, or changing the water in which the animals were maintained, was only slightly effective in stimulating spawning. Stimulation associated with the partial opening of the valves was more effective in this regard.

4. Possible explanations for the mechanism of spawning in mussels are discussed.

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AN ORIENTATIONAL RESPONSE TO WEAK GAMMA RADIATION[1]

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It is now well-established that widely different kinds of animals, when induced to move in any given compass direction on a horizontal plane in a presumed constant field, will tend to deviate, clockwise or counterclockwise, from this orientation. Variations in the direction and degree of this turning tendency appear to display all the major natural geophysical periods. The variations include also aperiodic ones of an unpredictable character. Furthermore, these tendencies at any given time vary with the geographic orientation of the animals.

In searching for factors which might be concerned in these orientational variations it has been discovered that mud-snails (Brown, Brett, Bennett and Barnwell, 1960; Brown, Webb and Brett, 1960; Brown, Bennett and Webb, 1960), planarians and paramecia (Brown, 1962a), *Volvox* (Palmer, 1962; 1963), and *Artemia* and *Drosophila* (unpublished observations in our laboratory) are not only extraordinarily sensitive to very small changes in magnetostatic field but are able, in addition, to resolve the horizontal vectors of very weak fields, including the earth's.

Other studies, with very weak electrostatic fields, have proven the snails (Webb, Brown and Brett, 1959; Webb, Brown and Schroeder, 1961; Brown, Webb and Barnwell, 1962) and planarians (Brown, 1962b) to be comparably sensitive to changes in ambient electrostatic fields, changes of the order of magnitude of those to which the organisms are steadily exposed in nature. Alterations in orientation are effected by ambient field changes of the order of magnitude of fractions of a microvolt per centimeter. For this force, as with magnetism, the animals appear able to resolve the horizontal vectors.

In the course of the above-mentioned studies evidence suggested that the organisms were also exhibiting changes in orientation with time and geographic direction which could not be accounted for in terms of the magnetostatic or electrostatic responsivenesses, either separately or jointly. There were indications that the organisms were responding continuously to one or more additional geophysical factors which were pervading the controlled experimental environment.

A preliminary exploration (Brown, Webb and Johnson, 1962) provided evidence that both mud-snails and planarian worms are able to perceive small changes in gamma radiation at intensity levels of the order of magnitude of the natural background radiation and, even more remarkably, to identify the direction

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of an experimental source. Initial tests, with the same results, had been made with radium gamma, but to obtain a simpler, or more homogeneous, radiation the study was, thereafter, pursued with radioactive cesium sources. This fact is mentioned only because it suggests a lack of specificity as to the effective gamma wave-length.

Methods and Materials

The discoveries that organisms are sensitively responsive to subtle geophysical forces which pervade the controlled environments of the type in which physiological responses are usually investigated, and that variations in one of these forces can alter response of the organism to another of them (Brown, 1962b), indicated the need for a new kind of approach to their investigation. Since one is dealing with kinds of identified forces whose regulation is difficult and often impractical, and since there are probably additional effective factors which have not yet been identified, a different method has been chosen from the usual one which is that of maintaining conditions constant for all factors other than the specific experimental one varied. This alternative method involves keeping constant the experimental changes to be tested and measuring the differences between experimentals and appropriate concurrent controls repeatedly over a sufficiently long period to assure reasonable randomization of effects on the response by the uncontrolled variables. For example, a one-month period of study at a fixed time of solar day would provide reasonable randomization of a lunar daily variation which is usually present, and at the same time would largely randomize the irregular variations in response associated, through correlated geophysical fluctuations, with passage of weather fronts.

It is important that the experiments be performed at a constant restricted time of day, or time of lunar day, since it has now been established that daily variations in response may be not only quantitative, but qualitative with sign of response changing in passing from one time of day to another. Thus, indiscriminate mixing of data collected over a wide range of hours of the day may lead to an erroneous conclusion that no response occurs when, indeed, one does.

Experience has shown, in addition, that one year of data, or simple multiples of a year, are required to randomize a highly significant persistent annual variation in influence of the experimental factors upon the organisms.

In brief, the nature of the response to changes in one specific kind of pervasive geophysical factor at any particular time, expressed as difference from concurrent controls, is itself being influenced by concurrent changes in other effective and uncontrolled pervasive factors. The specific contribution of any single factor can be resolved only in terms of its effect upon being tested repeatedly over a long enough time to render the effects of the other factors equal.

The common planarian worm, *Dugesia dorotocephala*, was used in this study. The worms were collected in the field on three occasions, once during each month, June, September, and October, 1962. The worms were kept in darkened containers in the laboratory and fed beef liver twice a week.

The apparatus and three-light field, with light sources behind, to right and vertically above the starting point of the worm have been described in some detail elsewhere (Brown, 1962a).

The mean path of 15-worm samples was determined as the average angular deviation from a path directly forward at the moment the worms crossed a circular arc whose center was one inch from the starting point of the worms.

The procedure was rendered as simple as possible, and arbitrary rules clearly defined, in order that wholly inexperienced observers could be used freely after very brief instruction and initial practice. For each experimental series on any given day five worms were used. The worms were swept one by one, repeatedly, to the starting point with a very fine sable-hair brush and oriented in a direction between the 0° and −10° axes on the underlying polar-coordinate grid. Numerous data had established that in such a three-light field the mean path of the negatively phototactic worms displayed a mode near −25° with an approximation to a normal distribution about this value.

As in all previous comparable studies of orientation of these planarians in such an asymmetrical three-light field, only worm starts which terminated in the worm crossing the one-inch arc between +5° and −50° were recorded. The path of each worm was recorded to the nearest 5°. On the most favorable days when the worms were moving well, less than 5% of the starts were failures. On the worst days, when many starts led even to immediate sharp U-turns, especially for some particular compass-direction for that day, as many as 50% to 60% of the starts had to be excluded on this same arbitrary basis.

The gamma sources comprised circular pieces of filter paper, four inches in diameter, upon which had been placed 24 μc. of Cs^{137} distributed uniformly in 8 spots equidistantly spaced on the perimeter of a 3½-inch circle, and a ninth spot in the center. The paper was sandwiched between plexiglass plates. The Cs^{137} plates were generously prepared in the radiation laboratories of the Marine Biological Laboratory, Woods Hole, Massachusetts, by Dr. R. J. Grabske of the Department of Physiology, University of Kansas, and Dr. Patricia M. Failla of the Argonne National Laboratory, Lemont, Illinois. The relation between plate distance in inches at right angles to the plate center and radiation intensity expressed as a multiple of background at Woods Hole is shown in Figure 1. This relationship was determined with the aid of a Tracerlab Monitor, #SU-3D. The sources, and dummy sources without the Cs^{137}, were placed in heavy envelopes and labelled X or Y by a person other than the worm-observer employing them.

The plates were hung on the outside of the wooden orientation chambers within which the worms were observed. The plates, unless otherwise specified, were placed so that their centers lay seven inches to right or left of the starting point of the worm paths. At this distance the intensity of gamma radiation at the position of the worms was about 6 times that of the natural background.

North- and West-Directed Studies

These observations were made on 36 afternoons, between 12:30 and 3:00 o'clock C.S.T., distributed as uniformly as was practical over the two-month period (59 days) from August 8 through October 5.

At each observation period, two observers, employing identically constructed apparatus, determined the average of 15 paths of worms under each of eight conditions. The conditions comprised four samplings of worms with the apparatus and initial path of the worms directed compass north, and four samplings with worms directed west. The order of presentation of north and west was varied

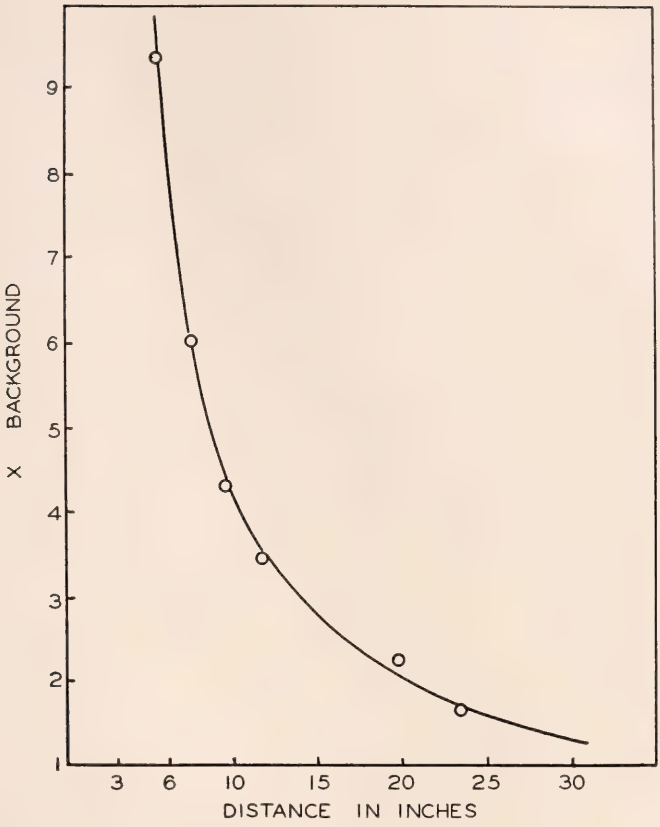

FIGURE 1. The relationship between distance from the sources used in this study and radiation intensity expressed as a multiple of the natural background radiation.

from one observation period to another. For each direction the mean path of the worms was determined under four conditions. These conditions were gamma source to right, gamma source to left, dummy source to right and dummy source to left, with the four offered in shuffled order; the observers were uninformed as to which envelope contained a source and which a dummy.

To learn whether the worms exhibited a measurable response to a Cs^{137} source, the mean path for each, $\text{gamma}_{\text{Right}}$ and $\text{gamma}_{\text{Left}}$, for each direction, was computed as the difference from the mean paths, in the same series of four, with the dummy to right and to left. It is evident that were there no response to the Cs^{137} source, the difference between the paths in the presence of the Cs^{137} source and those in the presence of the dummy controls should average zero. A significant difference from zero would indicate a responsiveness of the worms to the weak gamma sources.

Furthermore, if there was indicated by the preceding test that a response to the 6-fold increase in gamma radiation occurred, then any significant difference between the mean response when the gamma source was to the right and when it was the same distance to the left would indicate an ability of the organism to distinguish the direction of the source.

Results: The results of the two-month investigation are summarized in Figure 2 for north-directed worms and in Figure 3 for west-directed ones. Each point is the mean difference between the experimental (gamma$_R$ or gamma$_L$) and the average of the two controls (dummies) for the two experimental series of the same afternoon.

It is evident that for the worms initially directed northward in the earth's field, the gamma source on the left induced turning of the worms to the right and gamma to the right induced turning to the left. For the two average responses the values, $+1.157 \pm 0.389°$ and $-1.260 \pm 0.274°$, were obtained. Student's t in the two in-

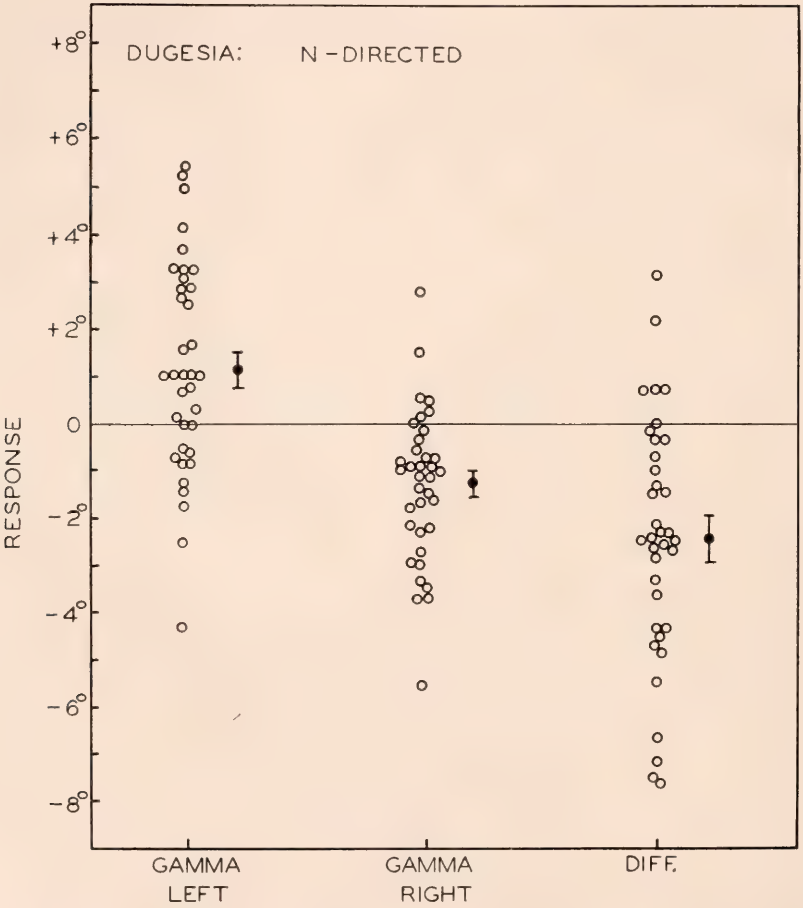

FIGURE 2. The distributions for N-directed worms of responses of 30-worm-path samples expressed as difference from concurrent controls in the earth's natural field alone. Response, expressed in degrees, is + to the right and − to the left. DIFF. is the difference between the two responses, to gamma$_R$ and gamma$_L$, for each of the the 36 experimental series taken separately.

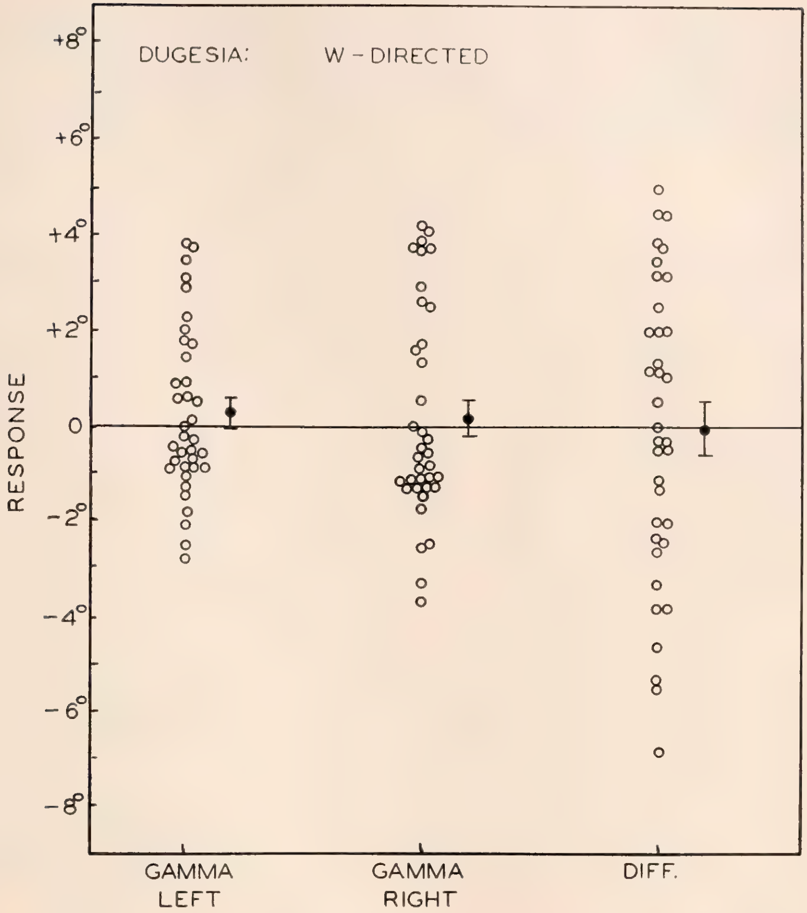

FIGURE 3. The distribution for W-directed worms of responses of 30-worm-path samples expressed as difference from concurrent controls in the earth's natural field alone (see Figure 2 legend).

stances, 3.03 and 4.69, respectively, with N in each case being 36, indicates high significance. Combining the two, the worms turned away from the gamma source, which ever side it chanced to be, with an average response of 1.208 ± 0.235°; t = 5.14, N = 72.

The data of each of the two observers, treated separately, indicated clearly this negative response. No reasonable doubt exists that the worms not only are able to perceive the presence of the gamma source, but to distinguish the direction of the source as well.

It is interesting, as seen in Figure 3, that when initially directed westward, the worms exhibit no similar orientational response to the gamma sources even though the experiment has, other than in compass direction, been conducted in exactly the same manner and at the same time as for the north-bound worms. Although no significant difference in mean paths between experimentals and controls in the west-bound worms is present, it is suggestive that with a probability of about 5% or less, the variance of paths with gamma to left is larger than variance with gamma to right in the north-bound worms and the corresponding difference reversed for the west-bound ones.

SOUTH- AND EAST-DIRECTED STUDIES

Even before the conclusion of the north- and west-oriented studies, other fully comparable studies were commenced involving south and east orientations. These were conducted over the period September 27 to November 2, inclusive. Responses to the gamma radiation were assayed on 26 different days distributed over the whole 36-day period. Three new observers were employed. These latter observers were completly uninformed about the nature of the previous results and, in fact, had no knowledge of the character of the problem. Sixty per cent of the observations for south and east were made by the three new observers; the remainder were made by the same observers who worked with north and west studies, still working uninformed as to the conditions obtaining at the time of their observations. Four identically constructed pieces of apparatus were used. The experiments were now performed over a wider range of afternoon hours, from 12 noon to 5 P.M., C.S.T., the greater time-span probably contributing in part to the slightly greater variability encountered.

Results: The results are illustrated in Figures 4 and 5. Each point represents the average response of 30 planarians to the gamma radiation, with 15 paths having in every case been determined by one person and the second 15 by another person. The second person was working either concurrently with the first, or following the first by a short interval.

As in the N and W studies, the results of each individual observer, in this instance now five, independently demonstrated the turning response of the worms away from the side of the gamma source when the apparatus was directed southward and the lesser, or no, response of this nature when E-directed.

Again, it was also clearly apparent that nothing related to the order in which the series was run contributed to the response. It was immaterial whether Gamma_L preceded Gamma_R in a series, or the reverse, or whether in a double series E preceded S, or the reverse. When directed southward, the worms turned significantly away from gamma on the left $(+1.403 \pm 0.458°;\ t = 3.06,\ N = 37)$ and away from gamma on the right $(-1.402 \pm 0.530°;\ t = 2.65,\ N = 37)$. Or expressed in another manner, the worms turned away from the gamma source, without regard to what side it was on, $1.402 \pm 0.363°$ $(t = 3.867,\ N = 74)$.

On the other hand, when the worms were directed eastward, the worms did not turn significantly away from the gamma source. For Gamma_L, the mean response was $+0.854 \pm 0.455°$; $t = 1.88$, $N = 39$. For Gamma_R, the response was $-0.124 \pm 0.526°$; $t = 0.24$, $N = 39$. For response away from the source, irrespective of the side the source was placed, the response of the E-directed worms was $0.489 \pm 0.346°$; $t = 1.41$, $N = 78$.

Combined results: The principal results are summarized in Table I. Taking all data, with no selection of direction of orientation nor particular side for the gamma source, the worms, on the average, clearly turned away from the gamma source. Whether one tests probability by means of student's t, which assumes a normal distribution of responses ($t = 5.08$; $N = 296$), or resorts to a non-parametric, Chi-square test with one degree of freedom, which makes the simplest possible assumptions ($\chi^2 = 36.9$), there is no reasonable doubt that the worms not only perceive the experimental introduction of the gamma source, but recognize the

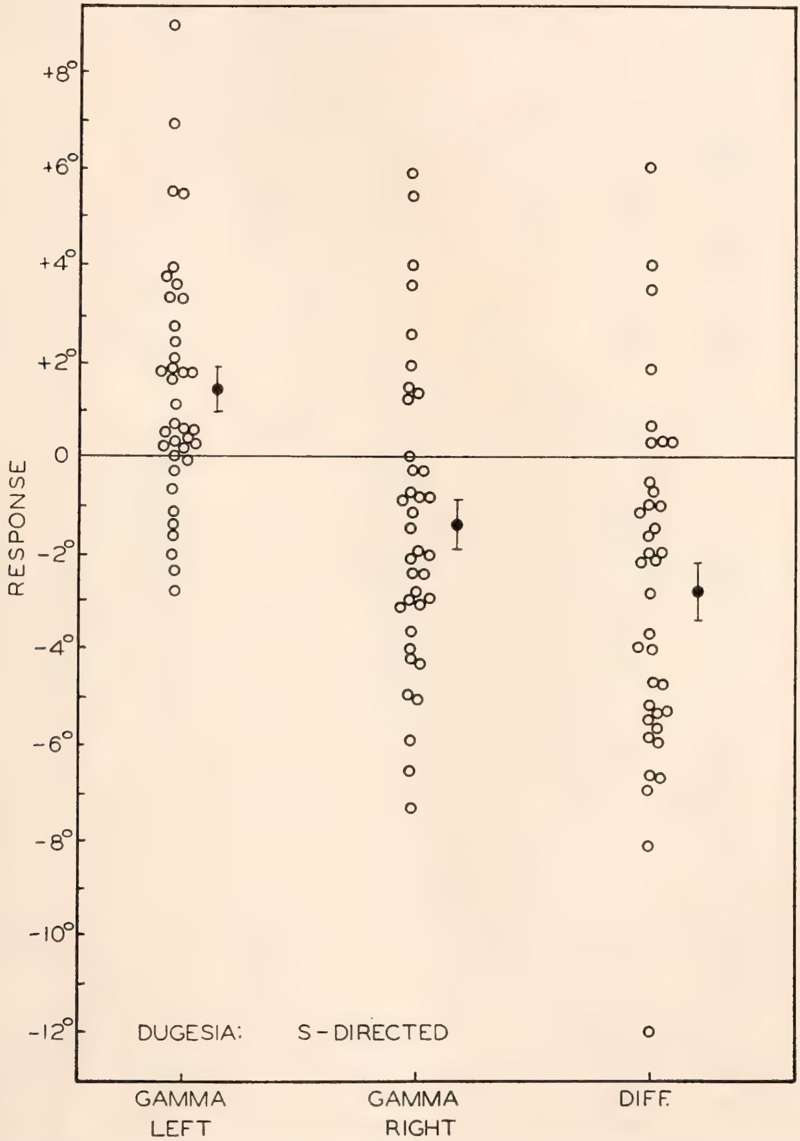

FIGURE 4. The distributions for S-directed worms of responses of 30-worm-path samples expressed as difference from concurrent controls in the earth's natural field alone (see Figure 2 legend).

side upon which it is placed. But further inspection of Table I confirms that this very highly significant response has been based almost entirely upon the avoiding response to gamma when the worms were crawling northward or southward in the earth's geographic field; only a small contribution comes from E-directed observations, and none from W-directed ones.

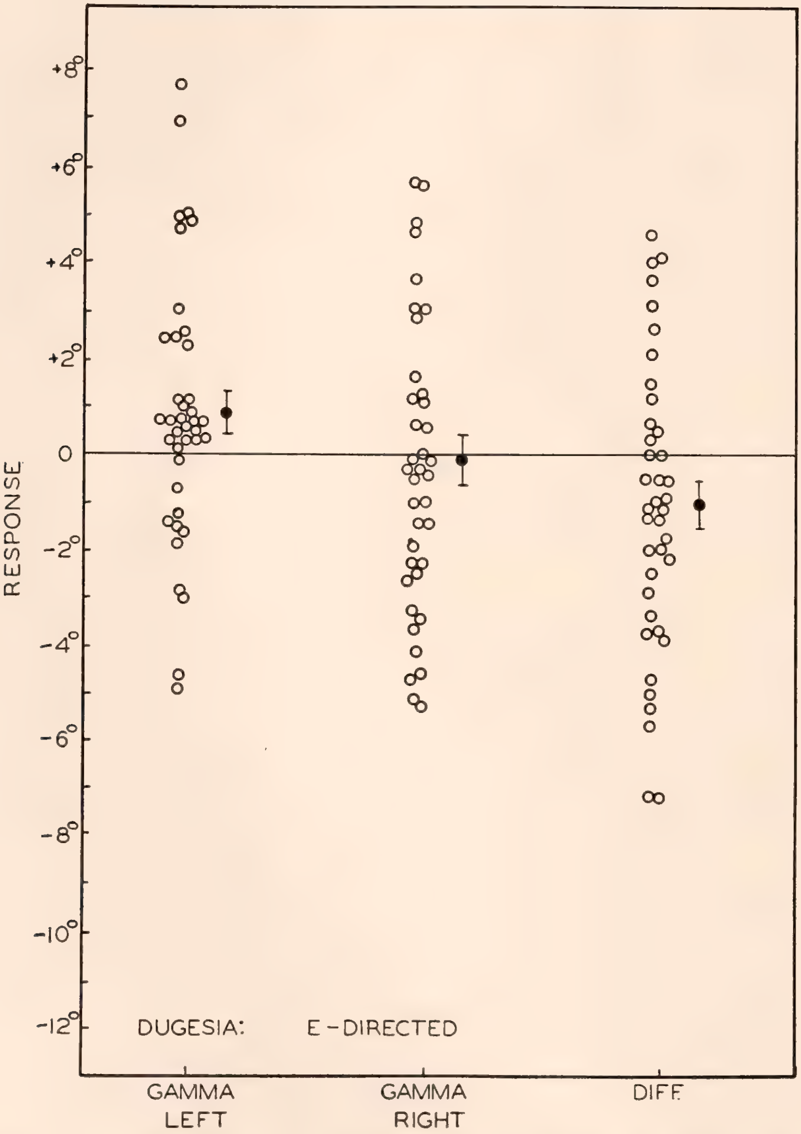

FIGURE 5. The distributions for E-directed worms of responses of 30-worm-path samples expressed as difference from concurrent controls in the earth's natural field alone (see Figure 2 legend).

TABLE I

Summary of gamma responses relative to geographic orientation

Direction	Response to gamma source	No. of samples of 30 paths	Number of cases		
			away	toward	neither
N	−1.208 ± 0.2352 $(t = 5.14)$	72	52	16 $(\chi^2 = 19.05)$	4
E	−0.489 ± 0.3455 $(t = 1.47)$	78	51	26 $(\chi^2 = 8.13)$	1
S	−1.403 ± 0.363 $(t = 3.86)$	74	54	18 $(\chi^2 = 18.01)$	2
W	+0.029 ± 0.238 $(t = 0.12)$	72	38	32 $(\chi^2 = 0.51)$	2
Combined N and S	−1.307 ± 0.216 $(t = 6.05)$	146	106	34 $(\chi^2 = 37.05)$	6
Combined E and W	−0.2402 ± 0.2125 $(t = 1.13)$	150	89	58 $(\chi^2 = 6.54)$	3
All directions	−0.781 ± 0.1538 $(t = 5.08)$	296	195	92 $(\chi^2 = 36.90)$	9

MONTHLY RHYTHM OF NORTH-DIRECTED WORMS

In an earlier published account (Brown, 1962a) a monthly rhythm in tendency of *Dugesia* to veer from a northward path was described to continue over about a 17-month period and to exhibit what appeared to be an annual variation in the form of the rhythm. From about September to about March there was a unimodal monthly fluctuation with maximum right-turning near full moon and left-turning near new moon. This became transformed about March or April into a semi-monthly fluctuation with maxima in right-turning near both new and full moons.

Observations on deviations in paths of north-bound worms have now been continued for about an additional year preceding, and concurrently with, the present gamma study. The results are seen in Figure 6. Confirming strikingly the earlier study, it is evident that a semi-monthly variation, with maxima in right-turning near both the times of new and full moon, occurred again during the warmer months, with the form of the fluctuation becoming converted into a unimodal monthly one in September. This latter type of monthly rhythm continued through the fall and winter, bearing the same phase relations to elongation of the moon that had obtained during each of the preceding two years except for an apparent tendency to revert to a transient, but typical, semi-monthly form of cycle in late January and through February. There was a return to a full-month cycle in March and onward past the dates of full moon and new moon in April (not included entirely in Figure 6). As with the earlier investigation of this monthly variation, the observations were made always between 8:30 and 11:00 A.M.

It is evident that the present study on responses of *Dugesia* to weak gamma radiation was being conducted during a period when this lunar rhythm was alternating between semi-monthly and monthly cycles.

A MONTHLY VARIATION IN RESPONSE TO WEAK GAMMA RADIATION

In an experimental series commencing on September 27, 1962, and extending through April 13, 1963, the influence of a series of strengths of gamma radiation on worm orientation was assayed, usually on four to six mornings a week. The gamma sources in this series were presented always on the right, or east, of worms moving northward.

Each of two observers on each morning determined the path of 15 worms under four strengths of the experimental gamma field, 9, 6, 3, and 1.5 times the background. The gamma field samples of 15 paths were alternated with control runs (with dummy sources) of 15 worms in the earth's field alone. The order of presentation of the four gamma strengths and whether the controls preceded or followed each gamma run was determined for each daily series by lot. Furthermore, although each observer could note during an experimental series what gamma strength was involved in each pair of samples, the observer was always uninformed as to which of a pair was control and which was gamma. There was thus full protection against any unconscious biasing of the results by an observer.

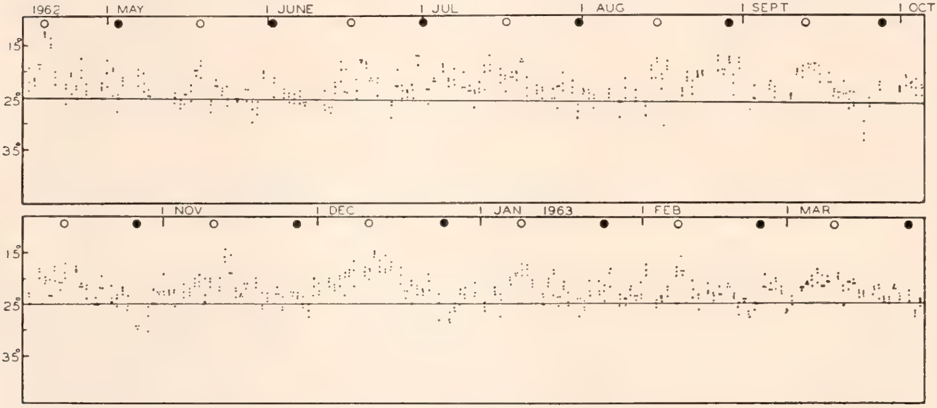

FIGURE 6. Variations in the character of the monthly orientation rhythm of north-bound *Dugesia* over nearly a year (see text).

The results which were obtained during the first 40 days are expressed as the mean difference in degree between all eight controls in the two daily series and all eight gamma fields, including the four different strengths. These results are illustrated as a function of calendar date in Figure 7A. A monthly variation is strongly suggested, with maxima in positive response occurring near the times of first quarter of the moon and the minimum occurring near third quarter. However, such a unimodal monthly fluctuation did not persist beyond this single cycle, but rather appeared to become converted abruptly to a semi-monthly one with maximum turning from the gamma source during a four-day period before both new and full moon and maximum turning toward the source during two days following these times. By December 4 three such semi-monthly cycles had clearly suggested themselves by inspection of the data. These data are depicted in Figure 7B, for the period October 20–December 4, in relation to the days of new and full moon. It is evident that during the four-day periods preceding the two moons there were 10

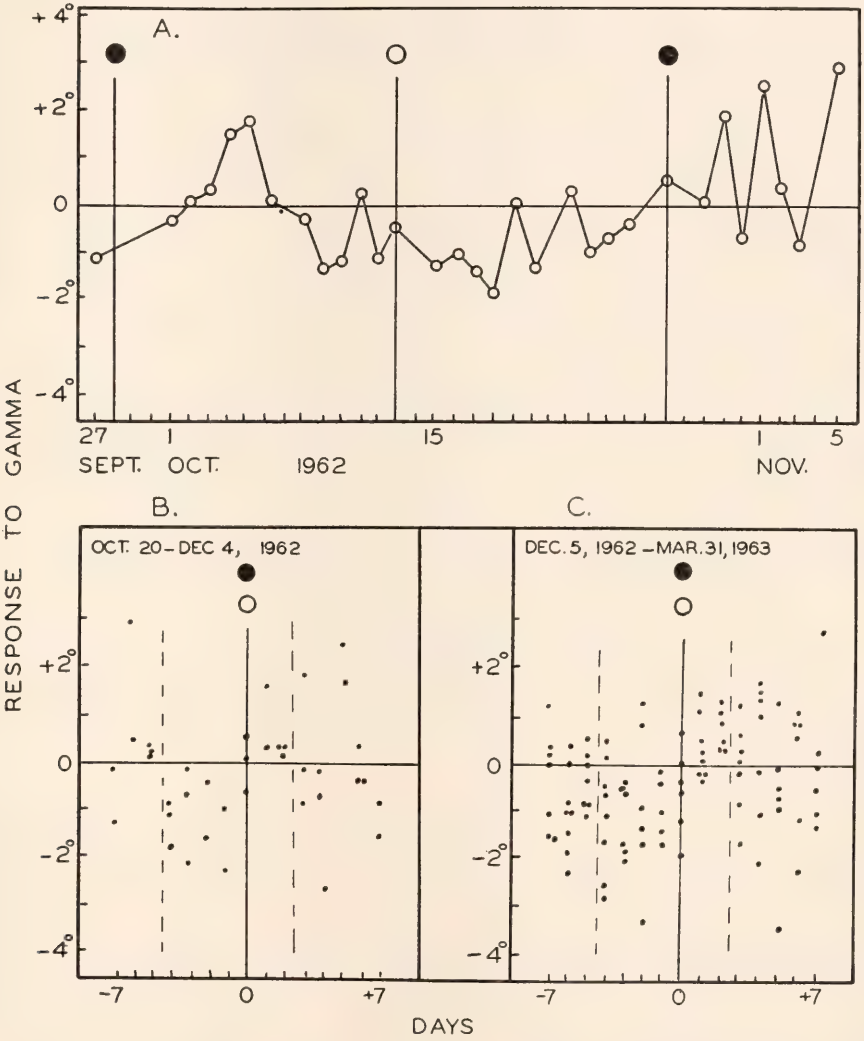

FIGURE 7. A. An apparent monthly fluctuation in response of *Dugesia* to all four strengths of gamma sources (from 1.5 × to 9 × background) presented on the right over the period from September 27 to November 5, 1962. B. A suggested semi-monthly fluctuation in response during three semi-monthly periods (October 20–December 4) with left-turning during four days before new and full moons, right-turning for two days afterwards, and high dispersion of responses for the remainder of the cycles. C. Confirmation for such a semi-monthly pattern derived from the study of an additional 8 consecutive semi-monthly cycles.

negative and no positive responses, and during the two days after these times there were 5 positive and no negative responses. During the remainder of these semi-monthly periods, the range of variation exceeded the total range of the first two periods but exhibited no consistent tendency toward either positive or negative responses.

Supporting the non-parametric estimations of probabilities, parametric consideration of these data indicated (1) the worms to be turning away from gamma sources during the four days before new and full moon with $P < 0.05$, (2) a difference between the mean for this interval and that of the two days after new and full moon with $P < 0.02$, and (3) the variance during the period from third to seventh day after new and full moon to be greater than that of either of the other two intervals with $P < 0.05$ in each case.

This hypothesis that the semi-monthly pattern had not occurred by chance was next tested over the period, December 5–March 31, which included 8 complete cycles. The results are illustrated in Figure 7C. Just as for the first three cycles shown in Figure 7B, the sign of the mean responses to the gamma fields rapidly altered from clearly negative to clearly positive in passing over the days of each new and full moon. The worms again turned during the second, and test, period statistically highly significantly to the left during the four-day period before the moons and to the right during the two-day interval afterwards. The parametrically determined significant probabilities were $P < 0.05$ for left-turning during the four days before the moons, and $P < 0.01$ for the difference between mean turning of this interval and that of the two days after the moons.

Equally apparent in both the initial three cycles and the additional confirmatory eight cycles is the exaggerated variability in response to the gamma sources during the interval from the third to seventh days after new and full moons or, in other words, as the moon's quarters are approached. The range exceeds that of both the positively and negatively responding periods combined. The variance is statistically significantly higher than for the positive period ($F = 4.33$; $P < 0.01$) but not, however, for the negative period ($F = 1.35$). It seems reasonable to postulate that this interval approaching the quarters of the moon is one in which there are continuing responses to the gamma field but that the response is sometimes positive and at other times negative.

The average form of the semi-monthly variation is illustrated in Figure 8A for all four strengths for the entire period of 13½ semi-months and in Figures 8B, C, D, and E for each of the four strengths of the gamma field taken separately. It is evident that in passing from the 1.5 × field to the 9 × one there is a progressive increase in the amount of turning away from the gamma sources during the quarterly periods preceding the new and full moons. Consequently, there is a progressive increase in the conspicuousness of the semi-monthly variation in response. This relationship between field-strength and response will now be analyzed.

RELATION OF RESPONSE TO GAMMA FIELD-STRENGTH

As described in the preceding section a total of 160 double series of the four gamma strengths were obtained between September 27, 1962, and April 13, 1963. In view of the demonstrable semi-monthly variations in the sign of the response of the worms to the gamma sources, the data were reduced arbitrarily to the ten

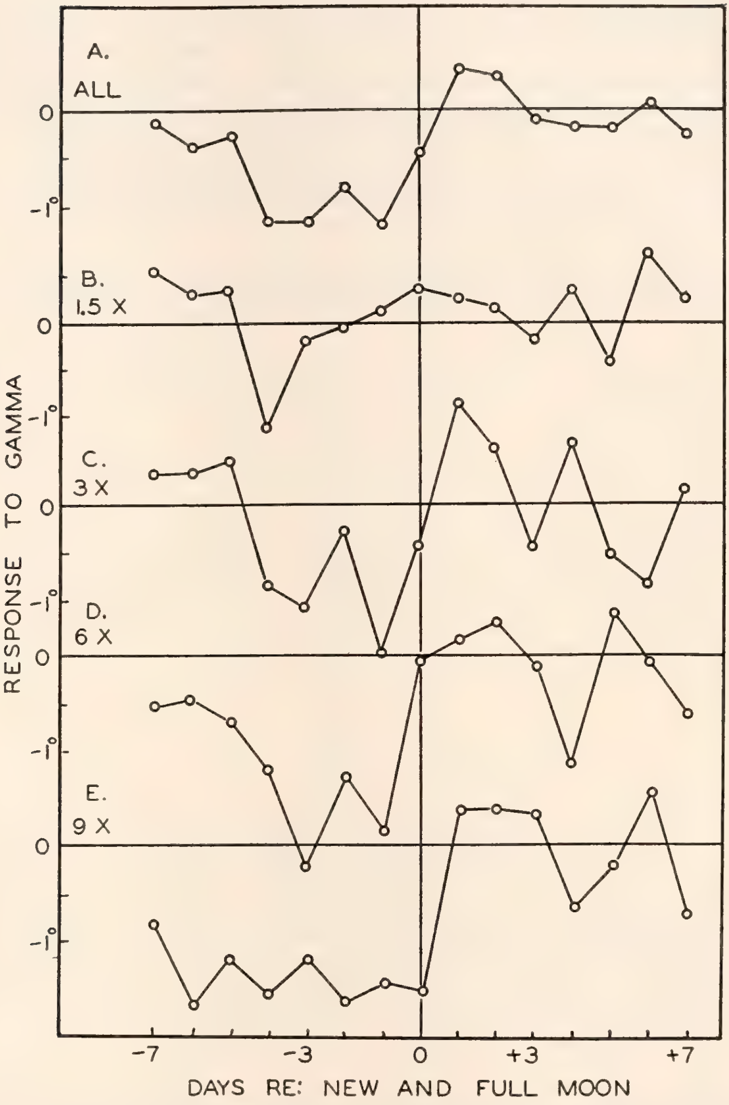

FIGURE 8. The mean apparent response to all four gamma strengths presented on the right in the morning as related to day of a natural semi-month. B, C, D and E. The same as above but for each gamma strength taken separately.

consecutive groups each containing 16 experimental double series. Since the data were obtained over about 13½ semi-monthly periods, the division of the data into ten groups assured that each group would include a reasonably sized sample of the large range of variation which was encountered. In Figure 9A are depicted the responses to the gamma sources for each of the four field-strengths expressed as the mean difference for each strength from the average of all the interpolated controls in that series. Each point, therefore, is the average difference of 480 worm-paths from 1920 control paths taken at the same times during the same experimental period. The small x's in the figure indicate the four actual arithmetical means. The solid line is the best straight line estimated by eye to include the four means and transect zero on the ordinate at 1 × on the abscissa.

It will be recalled that the gamma sources were always presented on the right. The mean overall response for all four fields is seen to be a negative one to the source. Thirty-one values indicated left-turning, 9, right-turning ($\chi^2 = 12.1$; $P < 0.001$).

It is evident from the data in Figure 9A that there was a graded mean response which was related to the field strength. The response was greatest for 9 × and least for 1.5 ×.

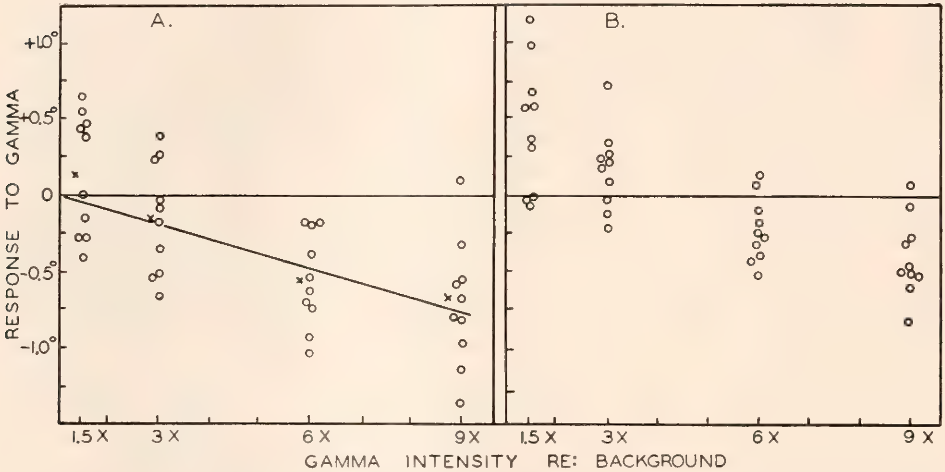

FIGURE 9. A. Difference in paths between planarians in the earth's field alone and in a field with a gamma source to the right in the morning at each of four gamma intensities expressed as multiples of background. Small x's within the figure are the means. Each point for each field strength represents the mean difference for 480 worm-paths. B. Same data as in A replotted as deviations from mean responses for the concurrently obtained four field-strengths, in order to reduce any correlated variation in the series which was a consequence of common controls, or secular trends, through the experimental period.

The method of reduction of the data, in which the response to each of the four gamma strengths in each sample series was computed as deviations from a common control, would obviously be expected to contribute a correlated variation among the responses to the four field strengths. This contribution, together with any biological alteration in responsiveness to the unilateral gamma field over the course of the seven months, was reduced by re-expressing the responses to each of the four strengths as deviations from the mean response for the four as a group in each sample period. These values are plotted in Figure 9B. That considerable variation was correlated in the data of Figure 9A is apparent from the reduction in dispersion of data for the three stronger gamma fields.

The solid linear regressional line drawn in Figure 9A appears to be about as good a fit to the data as the dispersion of the values permit. The values of Figure 9B treated as a linear correlation between angle of response and field strength yielded $r = -0.735$. The probability that this is not zero is < 0.001 ($t = 6.68$; $N = 40$).

The foregoing relationship could not be expected to obtain by chance were the worms either insensitive to the imposed weak gamma fields, or unable to distinguish the differences in strengths among these weak fields.

In considering Figure 9 it should be recalled that the response was an orientational one to gamma presented to the right or east. Although, for example, 1.5 × and 9 × involved a 50% and 900% increase, respectively, relative to total background gamma, the percentages were undoubtedly greater with respect to any east-west vector of background gamma radiation. A horizontal contribution to natural background gamma radiation would be expected to be relatively small in comparison with a vertical one.

There is a suggestion from the decentralization of the values that the response to the 1.5 × field may be sometimes negative and at other times positive. The negative cluster of data in Figure 9A was, in four out of five cases, obtained in the fall (the first five groups), and four out of five of the positive cluster of values were obtained in the winter. This suggestion, that to such weak gamma fields there may be an annual variation in sign of response, is being investigated further.

Additional suggestion that the response to the 1.5 × field includes sign reversals comes from Figure 9B. In this figure the responses of the worms to the four field strengths, treated as correlated variables, show substantially decreased ranges and variances for the three stronger fields but no comparable reduction for the 1.5 × field.

INFLUENCE OF ALTERATION OF ELECTROSTATIC FIELD ON GAMMA (6 × BACKGROUND) RESPONSE

This experimental series was performed 304 times by several observers working between 1 and 5 PM during the period from November 5, 1962, to April 15, 1963. Each afternoon run consisted of assaying 15 worm-paths under each of the following 8 conditions: 152 series were run *north-directed*—Gamma$_{\text{Right}}$ (1) 2 V/cm. electrostatic field in air with + to left and (2) equipotential plates as control); and Gamma$_{\text{Left}}$ (3) electrostatic field with + to left, (4) control; and Gamma$_{\text{Right}}$ (5) electrostatic field with + to right, (6) control); and Gamma$_{\text{Left}}$ (7) electrostatic field with + to right, (8) control. The other 152 series differed only in that they were *south-directed* always. The order of Gamma$_{\text{R}}$ and Gamma$_{\text{L}}$, ES field and control, and ES$_{\text{L}}$ and ES$_{\text{R}}$, was randomly varied. The time of day was the same as that of the initial experiments of the late summer and early fall when it was found the worms would turn away from the gamma source.

In Figure 10 are shown the differences between the mean path of the worms when the gamma source was on the right and the path with gamma on the left for the worms in the control or equipotential right-angle field (= "Diff" in Figure 2). Each datum is the average of the two groups of 15 paths in each gamma orientation during each single afternoon series.

It is evident from Figure 10A that, oriented *southward,* the worms turn further to the left when gamma is on the right than when it is on the left. The mean difference was −1.048 ± 0.2355° (t = 4.45, N = 152). On the other hand, when the worms were oriented *northward* (Fig. 10B), the worms did not, on the average, turn significantly away from the gamma plate (mean = −0.508 ± 0.276; t = 1.48, N = 152).

That the northbound worms were always responding to the gamma source, but sometimes positively and at other times negatively, is suggested by two observations: (a) the shoulders of the distribution are broader, and (b) the variance for the N-bound responses is significantly higher than the variance of the S-bound responses ($F = 1.37$; $N_1 = 152$, $N_2 = 152$; $P < 0.01$).

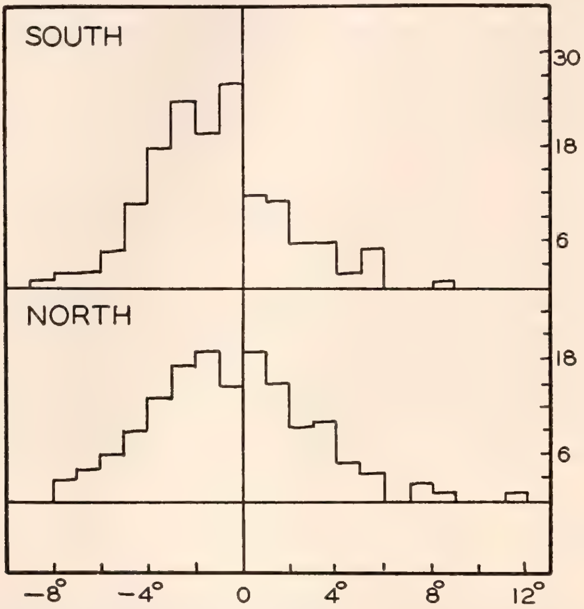

FIGURE 10. Frequency distributions of differences in path of southbound and northbound *Dugesia,* moving between large equipotential plates in the afternoon, when a gamma plate was to the left and when it was to the right (6 × background). Negative values indicate turning away from side of gamma source, positive values, toward source.

DISCUSSION

The results of this investigation add one additional environmental factor to the gradually increasing list of ones to which the living system is responsive. This one, like magnetism and electrostatics, pervades the usually controlled conditions of the physiological laboratory. Gamma radiation is highly penetrating. Furthermore, gamma radiation is a vector force, possessing direction as well as intensity. The sources of the gamma radiation contributing to the natural background are many and diverse and the radiation would be expected to possess at any given moment a specific three-dimensional pattern, just as does the natural environmental visible-light field.

This study has left no reasonable doubt that the planarians perceive not only the experimental change in gamma intensity, but distinguish field strengths, and the direction of the source, as well. Therefore, it would appear reasonable to presume that a living system is able to resolve and show responses to the detailed characteristics of the three-dimensional natural gamma field and to any variations in this field that may occur in nature. Differences in vector pattern with different geographical loci might be recognizable, as might also any alterations in the pattern related to phase angles in such natural geophysical cycles as the solar day, lunar day, month and year. In brief, this demonstrated remarkable sensitivity of the living organism to gamma radiation provides another potential source of information available to the organism to contribute toward refinements in its adjustment to the specific planet upon which it lives.

The mechanism by which the organism is able to respond to these very weak gamma fields is not immediately evident. Obviously, receptive mechanisms would gain no particular advantage by a superficial location on the body. These very low experimental intensities, of course, increase the production of ion-pairs throughout the protoplasmic system, albeit to a very low degree. It is, however, very difficult to imagine adequate sensitivity of the living system to enable it to differentiate the direction of the minute gradients in ion-pair formation, or in physical excitation, which would be a consequence of the reduction in intensity with increasing distance from the source. As is evident from Figure 1, the width of the planarian body (about 1 mm.), at a distance of about 18 cm. from the source, would permit only an extremely small intensity difference across the body. However, if this is truly the mechanism of directional resolution of the gamma source, the capacity of the organism to distinguish minute differences in intensity of gamma radiation becomes underscored.

This responsiveness to very weak gamma radiation displays some of the same types of characteristics which have been described previously for responses to very weak electrostatic and magnetostatic fields. For all three kinds of fields the character of the response has been shown to possess a "compass-direction effect" for organisms as different as snails and planarians. The response to gamma radiation, as with response to extremely weak electrostatic fields, appears to be maximum when the organism is oriented, during daylight hours, in a N–S axis and minimum when it is directed in an E–W one. The response to gamma, however, is in the same direction for both N and S orientations, while for electrostatic fields, the two responses are of opposite sign.

In common with response of planarians to very weak magnetic fields, the sign of the response to the gamma radiation is in natural conditions the same for both N and S orientations. Another similarity with magnetic response is evident. The response to gamma radiation in snails (Brown, Webb and Johnson, 1962) appears to exhibit a monthly variation. This suggests that the gamma responses are related to the biological-clock phenomenon. This study supports a conclusion that response of the worms to gamma radiation also displays solar-day, lunar-day, and perhaps annual variations. It is interesting to speculate that response to gamma radiation may contribute to the annual rhythm in compass-directional behavior in planarians reported earlier (Brown, 1962a) and now confirmed during this study.

The compass-directional differences in response of *Dugesia* to gamma radiation are very pronounced. The conspicuous responsiveness to the gamma radiation when the experimental gamma change involves an E–W axis, and the essential absence of response when the gamma axis is a N–S one supports the suggestion that the perception of changes in gamma pattern may be related to biological-clock and compass phenomena. The major periods of the biological clocks are related to rotation and revolution of the earth relative to sun or moon. The earth's rotation relative to sun and moon, is, of course, principally an E–W, and not a N–S, one. Therefore, it is plausible to speculate that this particular compass-directional sensitivity is a biological adaptation contributing to the recognition of the major geophysical cycles by this subtle means. This possibility is being explored.

SUMMARY

1. The common planarian worm, *Dugesia dorotocephala,* displays a significant orientational response to increase in Cs^{137} gamma radiation when the increase is no greater than 6 times background.

2. The worms are able to distinguish the direction of the weak gamma source, turning away from it, whether it is presented on the right or left side. The response sign is, therefore, the same as that of the response of these negatively phototactic worms to visible light.

3. There is a clear compass-directional relationship of the responsiveness to the experimental gamma radiation. A conspicuous negative response is present when the worms are traveling northward or southward in the earth's field with the gamma change in an east-west axis. No statistically significant mean turning response to the gamma radiation is found when the worms are traveling eastward or westward in the earth's field with the gamma change in a north-south axis.

4. The previously observed annual fluctuation in the character of the monthly orientational rhythm of north-directed worms has been confirmed in an additional year of study. During colder months, the rhythm is monthly; during warmer months it is semi-monthly.

5. There is a semi-monthly fluctuation in the response of *Dugesia* to weak gamma radiation during mid-morning hours, the worms turning away from the source for four days prior to new and full moon, and toward it for two days following new and full moon. The stronger the field strength, up to 9 times background, the larger the amplitude of the rhythm.

6. There is a direct relationship between intensities of gamma radiation between that of background and 9 times background, and the strength of the negative response of the worms.

7. Evidence suggests that the negative response of *Dugesia* to a gamma source may be modified by experimental alteration of the natural ambient electrostatic field.

8. Some possible biological significances of this remarkable responsiveness to gamma radiation, and its particular properties, are discussed briefly.

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ON THE PRESENCE OF A FEEDING HORMONE IN THE NEMATOCYST OF HYDRA PIRARDI

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Loomis (1955) reported that the feeding response in hydra is specifically stimulated by reduced glutathione oozing from the tissues of the prey animal after the penetration of the animal by hydra nematocysts. Recently, Forrest (1962) reported a lack of dependence of the feeding reaction in hydra on GSH. She cites numerous past publications which do not confirm the notion that hydra must feed on living animals containing GSH in their body cavities. In addition, she lists other compounds which mimic the effect of GSH in that they produce the typical tentacle movements and mouth-opening characteristic of the feeding response.

The present paper demonstrates: (a) that all scientists have not uncritically accepted "the GSH story," (b) that hydra is *not* necessarily stimulated to feed by substances issuing from the prey animal after nematocyst penetration, (c) that it is not known at the present whether GSH is or is not the specific stimulus to feeding in nature.

I. Substances Other Than Reduced Glutathione Which Will Stimulate the Feeding Reflex in Hydra

No attempt will be made here to review the papers which claim that substances other than GSH are capable of stimulating a feeding response in hydra. (See review of Forrest, 1962.) Suffice it to say that in addition to GSH, lactic acid, ascorbic acid, acetic acid, sodium acetate, ophthalmic acid, norophthalmic acid, papain, ficin, trypsin, quinine hydrochloride, plus other compounds, have been reported to elicit the response. In addition, we have observed the response after treating hydra with hyaluronidase, reduced methylene blue, dilute NaOH, and HCl.

From these observations it appears unlikely that GSH is the sole stimulus for a specific feeding reflex even in nature. Why do these diverse substances activate the feeding response?

Experiments conducted during the past two years have led us to conclude that in nature GSH may or may not be the sole stimulus of the hydra feeding response and that substances other than GSH (excluding GSH analogues) may cause a feeding response through a mechanism different from that activated by GSH.

First, let us consider the activation of the feeding reflex by GSH. When prey strikes the tentacles of the hydra, penetrant nematocysts puncture its tissues. Body fluids then ooze from the wound produced by the nematocyst thread. Loomis (1955) suggested that these body fluids contain GSH and that this compound stimulates the feeding response in hydra. Recently, Lenhoff (1961) demonstrated that hydra immediately begin the feeding response when they are placed in a 10^{-5} M

GSH solution. The mouths of these animals remain open for as long as 40 minutes. When washed in fresh culture water, they fail to respond maximally to GSH for 24 hours.

A slight modification of this experiment produced some instructive results. Forty hydra (*Hydra pseudoligactis*), starved for 48 hours, were placed in a Petri dish containing a 10^{-5} *M* GSH solution. The animals exhibited normal feeding response. After one hour all of the hydra had closed their mouths. To demonstrate that GSH was still present in the medium, five starving hydra were introduced into the same Petri dish. These animals immediately opened their mouths and began the feeding response. *Artemia* were then introduced into the Petri dish. Every one of the 40 animals which no longer responded to the GSH stimulus killed several *Artemia,* began a normal feeding response and ingested the *Artemia* normally. These results suggest that the contact of living *Artemia* with the tentacles of the hydra either reactivates the original GSH receptor site or stimulates another mechanism which is not related to GSH. In any case it has been demonstrated that hydra which no longer respond to a GSH stimulus *will* execute a feeding response when offered living *Artemia.* This observation casts doubt on the notion that GSH oozing from the nematocyst wound is the sole stimulus to the feeding reaction in hydra.

An explanation of substances other than GSH which will induce the feeding reflex has suggested that these substances operate through a mechanism which may be related only indirectly to the GSH mechanism. The substances tested in the present experiments were lyophilized crystalline trypsin (Armour Labs, Chicago, buffered to pH 6.3, employed in a concentration of 0.1 mg./ml.), hyaluronidase (Nutritional Biochemicals, Cleveland, buffered at pH 6.2, concentration 1 mg./ml.), lactic acid (10^{-4} *M,* pH 5). *Hydra pseudoligactis* which had been starved for 48 hours were introduced by means of microforceps into 1-ml. test solutions and observed with a binocular dissecting microscope at 20 × or with a compound light microscope at 100 ×.

Five hydra were placed in the test solution in a depression slide and observed at 100 ×. Trypsin, hyaluronidase, and lactic acid stimulated stenotele discharge from the tentacles. These chemicals appeared to lower the threshold necessary to stimulate the discharge of this particular nematocyst type. For instance, a hydra in a trypsin solution discharged as many as three dozen stenoteles from a limited area of a single tentacle when the tentacle contacted debris. Moreover, when one tentacle of the hydra contacted another tentacle there was a nematocyst discharge between the tentacles, and the hydra discharged stenoteles into its own tissues. Often the tentacles stuck to one another because of the nematocyst discharge from an adjacent tentacle, and a muscular exertion was necessary before hydra could separate its tentacles. Another reaction was also evident. If the hydra curled a single tentacle so that the extremity of the tentacle contacted more proximal regions of the same tentacle, there was a prompt discharge of stenoteles. Finally, nematocyst discharge was seen in tentacles which were not stimulated by either the glass slide or another tentacle.

Continued observations revealed that the hydra never opened their mouths nor began the feeding reaction until several stenoteles were discharged. It appeared that perhaps nematocysts piercing the tissues of the hydra itself were producing wounds through which GSH in the epithelio-muscular cells of the hydra could pass into the test solution and stimulate the feeding response. More will be said concerning this point later. It must be stressed that animals which were placed in a depression slide containing normal culture medium (Versene-bicarbonate solution—see Loomis and Lenhoff, 1956) did not discharge excess stenoteles either when the tentacles contacted each other or when they contacted the bottom of the slide.

In order to quantitate the increased stenotele discharge in test solutions, the following experiment was conducted. Five experimental animals, starved for 24 hours, were placed in 1 ml. of each test solution and examined. After these animals opened their mouths and were passing their tentacles through the mouth opening into the enteron, they were removed from the test solution with microforceps and placed on a clean glass slide. Five drops of a 10% alcohol solution (which paralyzes the animals and permits the investigator to count discharged stenoteles) were placed on the hydra, and a very light squash preparation was made by placing a coverslip over the animals. This squash preparation was then examined at 440 × and the number of discharged stenoteles was recorded. To ensure that excess stenoteles were not discharged in response to the 10% alcohol solution or the pressure of the coverslip, a control group of hydra was taken from the normal culture medium, placed in the alcohol solution and examined. The results of this experiment are seen in Table I.

TABLE I*

Culture medium	% of animals which opened mouths and carried on normal feeding reflex	No. of discharged stenoteles. Average for 5 animals
1. Normal	0%	17
2. Trypsin	100%	64
3. Hyaluronidase	80%**	36
4. Lactic acid	100%	50
5. GSH	100%	18

* It must be remembered that the number of stenoteles recorded in these experiments was probably less than the number which had been discharged in test solution because several discharged stenoteles were perhaps lost when the hydra was transferred from the test solution to the glass slide.

** One hydra did not respond to the hyaluronidase solution. Subsequent examination revealed that this animal released only three stenoteles. This figure significantly lowers the average number of stenoteles which were discharged for the group of 5.

It will be seen that the two enzymes and lactic acid stimulated a stenotele discharge which was at least double that of animals in normal culture medium or those in a GSH solution. Animals which were placed in a GSH solution in a depression slide and viewed during the feeding reflex at 100 × did not release stenoteles; limited stenotele discharge occurred only when these animals were crushed under a coverslip in an alcohol solution. Animals placed in lactic acid and hyaluronidase solutions did not open their mouths within 5–15 seconds as did the hydra placed in a GSH solution. As long as 30 seconds–two minutes elapsed before the feeding reaction occurred. Animals placed in a trypsin solution opened their mouths after 25 seconds to one minute. The response here is slower than that of animals in a GSH solution but faster than that of animals in hyaluronidase or lactic acid. These results may indicate that trypsin stimulates a much greater stenotele discharge than either hyaluronidase or lactic acid.

These results suggest that substances other than GSH or its analogues might stimulate the feeding reflex, not through an activation of a GSH receptor in hydra, but by stimulating stenotele discharge which ultimately results in a release of GSH from the tissues of the hydra itself. To test this hypothesis the following experiment was conducted. It is well known that strong acids or alkalis stimulate nematocyst discharge. Balke and Steiner (1959) were able to stimulate a feeding reaction in hydra with acetic acid. In our laboratory we commonly stimulated nematocyst discharge with dilute NaOH. Thus, if NaOH elicited a feeding reaction in hydra, then more support would be given to the hypothesis that stenoteles contain the feeding hormone.

Fifteen hydra starved for 48 hours were placed into a Syracuse watchglass containing 10 cc. 10^{-5} *M* NaOH. Within one minute three of these animals had opened their mouths and were carrying out a perfectly normal feeding reflex. The tentacle movements were well-coordinated, the mouth opened in a manner similar to that observed when hydra were placed in a GSH solution. This is not the "gaping" that Lenhoff (1961) has reported when hydra are placed in a solution of noxious compounds. The remaining 12 animals, although exhibiting tentacle movements characteristic of the feeding response, had not opened their mouths after one minute. At this time the tip of a thin dissecting needle was dipped into a solution of 10^{-2} *M* NaOH. The needle was then introduced into the culture medium and placed between the tentacles of animals which had not yet opened their mouths. The tentacles of these forms immediately began a more active feeding response and within a few seconds the mouths opened. By successively dipping the needle into concentrated NaOH solution and stimulating hydra which had not opened their mouths, we were able to stimulate mouth-opening in 12 of our 15 test animals. These animals were then placed in normal culture medium and were all in a healthy condition the following day.

II. Permeability Properties of the Nematocyst Capsule

These results could mean that substances other than GSH evoke a feeding response by stimulating a nematocyst discharge which causes the hydra to penetrate its own tissues with stenoteles, thereby introducing GSH into the surrounding medium. Further observations on the permeability of the nematocyst capsule forbid this conclusion.

Methylene blue readily enters and remains for several days within the stenoteles of the intact hydra. However, isolated, undischarged nematocysts when stained with methylene blue readily lose the stain when transferred to a fresh culture medium. Also, fully developed nematocysts in isolated cnidoblast cells similarly lose the dye to the surrounding medium when removed from the methylene blue solution. In short, only the intact hydra or portions of intact animals retain methylene blue within the nematocyst capsule.

These observations suggested that once the nematocyst thread had everted and projected beyond the cnidoblast cell, the contents of the nematocyst capsule which had not been discharged through the end of the thread might leak from the butt of the exposed capsule. The contents of the capsule of the stenotele when liberated into the surrounding medium might be capable of eliciting a feeding response. In this case the "environmental hormone" postulated by Loomis would be located in the tissues of the hydra itself.

III. STIMULATION OF THE FEEDING RESPONSE WITHOUT CHEMICAL OR MECHANICAL STIMULATION

In order to test this hypothesis, it was necessary to elicit a feeding response in hydra without relying upon chemicals applied to the external medium. This was accomplished by employing a method of nematocyst discharge recorded by Kline (1961) who employed electric shock to stimulate a massive nematocyst discharge.

In a typical experiment, four *Hydra pirardi,* starved for 24 hours, were placed in a drop of distilled water on a slide. A 72-volt shock from 12 6-volt batteries was applied for a period of one second. These hydra immediately were removed with microforceps and two normal individuals were placed in the drop of water. These animals promptly opened their mouths and began a normal feeding response. After a few minutes two more animals were introduced and they too began the feeding response. This maneuver was continued until eight animals were introduced into the medium. The feeding responses lasted for as long as 30 minutes. Several of the animals began to spread their mouths over the bottom of the slide in an attempt to devour it.

Similar results were obtained when excised tentacles were shocked in a drop of water. These tentacles were well separated so that they could not come into contact with one another and were observed during the shock to insure that they did not curl and come into contact with themselves. After one second of electrical stimulation the tentacles were immediately removed and ten hydra which were subsequently introduced into the solution began the feeding response. These results suggest that a substance within the nematocyst capsule itself stimulates a feeding response in hydra.

One question comes to mind. Burnett (unpublished observations) has observed that hydra may discharge only a few stenoteles to subdue a prey animal: is this discharge sufficient to stimulate a feeding reaction? The answer is "yes," provided the proper mechanical stimulation is present. We have confirmed the observations of Lenhoff (1961) that hydra which remain for long periods of time in a GSH solution eventually close their mouths. However, it has been demonstrated that if these same animals are stimulated by teasing their tentacles with microforceps or transferred to a slide with a pipette they will open their mouths and begin a feeding response.[1] This explains why hydra which have habituated to a GSH solution will readily accept and devour *Artemia.*

We have demonstrated that if a 48-hour starved hydra is picked up with a pipette and dropped upon a slide, as many as 20 stenoteles are discharged. These animals do not open their mouths but will wave their tentacles in a manner which resembles that of the feeding response. However, if the tentacles of these individuals are stimulated by rubbing microforceps along their length, mouth-opening invariably follows. The mouth will remain open for varying periods of time, depending upon the number of nematocysts discharged. When hydra captures a prey animal, it invariably brings the animal into contact with the mouth. Once the mouth opens, the prey is invariably ingested.

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[1] Recent experiments with *H. littoralis,* obtained from Lenhoff, have demonstrated that unlike *H. pirardi, H. littoralis* will not respond after it is habituated to GSH.

IV. Specificity of Glutathione in the Feeding Reflex

All of our experiments indicate that any substance which stimulates nematocyst discharge will stimulate the feeding reflex. Acids and alkalies are traditionally employed in classrooms to stimulate nematocyst discharge. However, most of these materials are noxious and if not used in extremely dilute solutions will cause the death of the animal. From the foregoing experiments it appears that lactic acid, ascorbic acid, quinine hydrochloride, hyaluronidase, trypsin, acetic acid, sodium hydroxide, electric shock, glutamic acid, sodium acetate, etc., stimulate nematocyst discharge and it is this discharge which evokes the feeding reflex, not the chemicals themselves. Other substances which stimulate the feeding response, such as beef broth, egg white, etc., may also stimulate a nematocyst discharge. At the present time, it is not known whether the factor within the nematocyst capsule is GSH.

V. Evolutionary Speculations

Loomis (1955) stated that hydra feed upon only those animals which liberate sufficient quantities of GSH after penetration by the hydra nematocyst. Loomis suggests that GSH would be found in this high concentration only in those forms which possessed a fluid-filled body cavity (*e.g.,* annelids). Upon puncture of these forms by nematocysts, fluids containing GSH would apparently ooze from the wound. For this reason hydra feeds, according to Loomis, only on forms such as annelids and arthropods whose coelom and haemocoele, respectively, would represent a fluid-filled body cavity.

First we agree with Forrest (1962) that hydra are not forced to feed only upon members of certain select phyla. We have found, for example, that a brown hydra (*Hydra oligactis*) may be dried in a desiccator for 48 hours, and the tiny dried piece of tissue will be ingested if dropped into the middle of the circlet of tentacles surrounding the mouth of a hungry animal of the same species. We also have observed that the brown hydra (*Hydra pirardi*) will kill and ingest a species of European green hydra (*Hydra viridis*). It is impossible to culture these two species in the same dish for this reason.

It is our contention that the hydra is not restricted to diet by the presence of a body cavity in the host animal, but depends on whether or not the prey organism elicits a stenotele discharge when it contacts the tentacles of the hydra. We would be very surprised indeed if substances like egg white, dried hydra, protozoans, and even mud, all of which have been claimed to elicit the feeding response, did not stimulate a discharge of stenoteles upon contact with the tentacles. Mechanical stimulation, coupled with stenotele discharge, will elicit a feeding response if only a few stenoteles have been discharged.

This observation explains perhaps how primitive coelenterates were able to feed with nematocysts. If one postulates that a body cavity must exist in the prey organism before the hydra can feed, then one may ask, what was the source of food materials for coelenterates before the higher coelomate phyla evolved? If one postulates that the "hormone" which stimulates the feeding response evolved within the capsule of the stenotele, then any animal, irrespective of body cavity, which stimulated nematocyst discharge could presumably be ingested.

It is not surprising that Loomis presented evidence that hydra does not feed upon other hydra or upon flatworms. First, many flatworms do not stimulate nematocyst discharge when placed on the tentacles of hydra. For example, we have never been able to elicit a feeding response from *Hydra pseudoligactis* by offering this form *Dugesia* sp. Moreover, most hydra will not discharge nematocysts against other species of hydra. In our experience only the green hydra will stimulate nematocyst discharge from *Hydra pirardi*. It is not inconceivable, however, that similar relations might exist between certain species of brown hydra.

At the present time, the only coelenterate which we have studied in terms of the feeding response is the common hydra. We urge that investigators study the feeding mechanism in marine forms in light of nematocyst discharge. Care must be taken to insure that the type of nematocyst discharged is the one employed by the animal to pierce the prey animal, not specialized nematocysts employed in locomotion, defense, etc. Once nematocyst discharge has taken place, then the tentacles should be immediately stimulated mechanically.

VI. Hydra Without Nematocysts

From the foregoing observations it might be concluded that a hydra which lacks nematocysts is incapable of exhibiting a feeding response. This is not the case. If the hypostome and tentacles are removed from a hydra and then the tentacles trimmed off at the junction with the hypostome, the isolated hypostome will not open its mouth when stimulated with lactic acid or hyaluronidase, both of which stimulate nematocyst discharge. However, these isolated hypostomes will respond for a short period of time to GSH which does not specifically stimulate nematocyst discharge.

This gives us strong reason to believe that GSH does not act directly through nematocysts in eliciting a feeding response. In fact, it is conceivable that GSH is actually present in the capsule of the stenotele and is still the compound ultimately responsible for the feeding response. It would be a precarious situation indeed for the hydra to depend upon the presence of a single molecule in the prey animal in order to feed. However, if this molecule were built into the animal's own system, it would be of definite selective advantage to the animal. On the other hand, it is possible that compounds other than GSH which are capable of eliciting a feeding response are located in the nematocyst capsule.

Summary

1. It is not necessary to postulate an environmental hormone in order to explain the feeding response in hydra. Since nematocysts which are removed from the tissues of the hydra do not retain small molecules (methylene blue) which they readily accept and bind in the intact animal, it is suggested that during nematocyst discharge, in addition to the introduction into the prey of a toxic material, a certain

substance or substances leak from the butt of the nematocyst capsule and diffuse into the surrounding medium. This substance(s) is capable of stimulating mouth-opening when it comes into contact with the hypostome. Dilute concentrations of the substance which will not elicit a feeding response are found to do so if the tentacles are stimulated mechanically. This explains how a few stenoteles, when discharged into an actively wriggling prey animal, are sufficient to stimulate mouth-opening.

2. Although there is evidence that GSH is not the specific evocator of the feeding response in hydra in nature, more thorough studies must be conducted. All of the materials which stimulate a feeding response and lack GSH may be shown to stimulate nematocyst discharge, and as we have suggested, the nematocyst may contain GSH.

3. It is suggested that for *H. pseudoligactis* and *H. pirardi,* at least, the term "environmental hormone" be dropped. If this primitive hormone does exist in the capsule of the stenotele the term retro-hormone might be more appropriate.

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CONTROL OF FLASHING IN FIREFLIES. II. ROLE OF CENTRAL NERVOUS SYSTEM

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In our preceding paper (Buck and Case, 1961, hereinafter referred to as "FF-I") we showed that many responses of the firefly lantern to electrical stimulation are analogous to those of conventional neuro-effectors such as striated muscle. In that paper we did not attempt to distinguish between central and peripheral nervous mediation in the excitation process. In the present investigation we have explored the roles of brain and cord more specifically. Some of the data have been summarized in abstracts (Case and Buck, 1958 and others cited in FF-I). We acknowledge with pleasure the assistance of Mr. Frank Hanson in the experimental work, and of Dr. Seymour Geisser in the statistical analysis in Section 4C.

MATERIALS AND METHODS

We studied adults of the lampyrid fireflies *Photinus pyralis* from Maryland and Iowa, *Photinus marginellus* and *Photinus consanguineus* from Woods Hole, *Photinus punctulatus* from Iowa, and the common photurid (*Photuris versicolor*?) from all three localities. Males were used exclusively except for *Photuris*.

The lantern consists of two thin photogenic organs, one in abdominal segment 6 and one in 7. Each organ occupies most of the ventral surface of its segment, just inside the transparent cuticle.

The central nervous system comprises brain and suboesophageal ganglion in the head, and a ventral cord consisting of three ganglia in the thorax and seven in the abdomen. The gross innervation of the lantern is derived from abdominal ganglia 4–6, each segmental photogenic organ receiving nerves from at least two ganglia (Hanson, 1962).

Stimuli were delivered by Grass S-4 stimulators via r-f isolation units and electrode pairs of 0.005-inch bare silver wire. The wires were placed 1–3 mm. apart, directly in photogenic tissue unless otherwise specified. Light emission was detected by a photomultiplier tube modulating one channel of a Tektronix 502 dual beam oscilloscope and photographed together with a second (stimulator) trace. Since all records are of multicellular responses and since no measurements were made of tissue resistance, nominal stimulus voltages have significance only as indications of the relative magnitudes of current flux under different experimental conditions. Further details are given in FF-I.

Action potentials were picked up with electrode pairs of 0.003-inch bare platinum-iridium wire. After amplification by a Grass P-6 amplifier the potentials were displayed on the oscilloscope and photographed together with the output signal of the photomultiplier monitoring light emission.

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RESULTS

1. *Spontaneous luminescence: multiple flashing*

In single-flashing species or individuals successive spontaneous flashes are often very uniform in intensity and frequency, implying a correspondingly regular central nervous signal (*e.g.,* FF-I, Fig. 1). In some species each of the regularly repeated spontaneous flash episodes consists of several partially fused subflashes, implying a more complex excitation. Figure 2 illustrates a triple flash of the male of the Woods Hole *Photuris,* in which the mean inter-peak intervals are roughly 40 and 60 milliseconds (msec.). In the triple flash of the female of the Maryland *Photuris* the inter-peak intervals are both about 85 msec. (Fig. 8 of Hastings and Buck, 1956). Triple flashes with interflash intervals of the order of 60 and 100 msec. were recorded from a small Woods Hole *Photinus* (FF-I, Fig. 7).[2] In most of these instances the intervals between sub-peaks are of the same order as those between successive peaks in the sawtooth luminescence induced by repetitive electrical stimulation at about the limit of 1:1 response (FF-I).

2. *Delayed flashing*

In individuals with central nervous system intact, supernumerary flashing is common after intense stimulation, particularly by trains of such high frequency that the animal has not been able to respond separately to each impulse. For example, in the sequence of *Photuris* responses shown in Figure 1, the major flashes during stimulation at 50 pulses/sec. were at first about 95 msec. apart, or one response to about every fifth stimulus. Some flashes had shoulders indicating a frequency of at least 30/sec., which is considerably above the 20/sec. limit of maintainable 1:1 response to electrical stimulation previously found in this species (FF-I). The flashes soon became less regular and intense and finally merged into a dull glow that died out soon after the stimulus train terminated. Then, more than 300 msec. later, flashing resumed. Like the response during stimulation, this delayed episode showed a major flash frequency of about 10/sec. about four times as high as the maximum rate of spontaneous flashing in intact specimens. Such episodes, each lasting a few hundred milliseconds, may recur at irregular intervals for over five seconds.

The brain is not essential to all post-stimulatory flashing. A decapitated firefly or even an isolated lantern excised with ganglion can often be sent into immediate repetitive activity by a single stimulus after several seconds of rest, and the stimulus need not necessarily be especially intense, particularly if the tissue has been primed by vigorous prior stimulation. For example, in a single segmental organ of *Photuris* that had first been thoroughly aroused by a succession of stimulus trains

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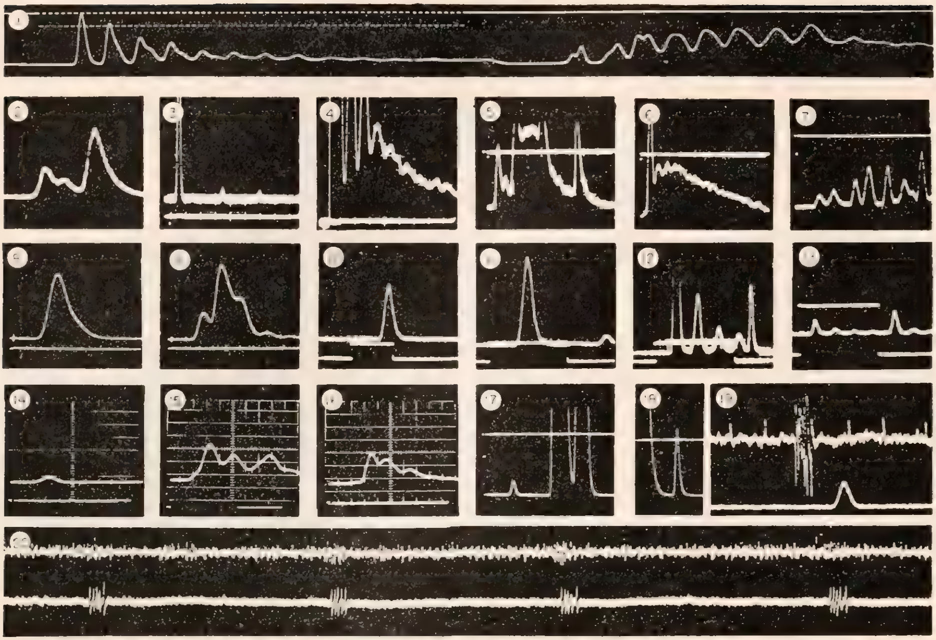
[2] Because of a mix-up in the records from two very similar small species of Woods Hole firefly, it is necessary to report that Figure 43, in FF-I, should refer to *Photinus marginellus* and Figure 50 to *Photinus consanguineus,* and that the species identifications for Figures 7 and 49 are uncertain. However, the phenomena illustrated were valid regardless of species responsible.



Figures 1–20. (In these oscillographs the time scale is from left to right and is given as S = entire width of picture in milliseconds. Figures identified "As X" refer to the same individual as that of Figure X. Some figures are slightly retouched.) (1) Woods Hole *Photuris,* male, electrodes in head. Responses to train of shocks of 10 m.sec./8 V., 50/sec., with post-stimulatory flashing. S = 2850. (2) Woods Hole *Photuris,* male, intact. Spontaneous flash. S = 270. (3) Iowa *Photuris,* male, isolated lantern. Response to single shock of 1 msec./5 V. S = 1950. (4) As 3, except 1 msec./50 V. Much reduced vertical amplification. S = 1950. (5) *Photinus marginellus,* male, intact. Response to single shock of 5 msec./40 V. S = 5000. (6) As 5, after decapitation. Vertical amplification reduced to 1/5. S = 5000. (7) *Photinus consanguineus,* male, decapitated. Response to single shock of 10 msec./7 V. S = 1000. (8) *Photinus punctulatus,* probably male. Response to single shock of 3 msec./9 V. S = 500. (9) As 8, but 2 msec./40 V. S = 500. (10–12) Woods Hole *Photuris,* male, isolated organ. Responses to single shocks of 100 msec./2.5 V., 200/2.5, and 400/2.5. S = 350, 350, 675. (13) As 10, after further dissection. Response to 400 msec./8 V. S = 675. (14) *Photinus marginellus,* male, decapitated. Response to single shock of 10 msec./15 V. S = 1100. (15) As 14, except 500 msec./30 V. S = 1100. (16) Next response to 15, but to 10 msec./15 V. S = 1100. (17) As 1, but 5 msec./8 V. S = 475. (18) Woods Hole *Photuris,* female, electrodes in head. Response to single shock of 5 msec./5 V. shortly after decay of spontaneous flash. S = 425. (19) Woods Hole *Photuris,* male, intact. Action potential volley from anterior segment of lantern (upper trace) and subsequent spontaneous flash (below). S = 440. (20) Woods Hole *Photuris,* male, intact. Concurrent action potentials from cord between 5th and 6th abdominal ganglia (above) and from surface of 6th segment light organ (below). Four photic volleys included. S = 2150.

close to its limit of 1 : 1 response, a very weak shock sufficed to induce three flashlets after the large primary response (Fig. 3), whereas a stronger one induced a paroxysm of brilliant high-frequency flashing lasting more than 2 seconds (Fig. 4). Figures 5 and 6 illustrate similar irregular luminescence in *P. marginellus* before and after decapitation. Repetitive firing was also induced in *P. consanguineus,* with a major periodicity of about 125 msec. (Fig. 7).

Transition to multiple flashing of a seemingly more regular type than the above can sometimes be induced simply by increasing the strength and/or duration of the stimulus shock. For example, by doubling the voltage the specimen producing the flash shown in Figure 7 was induced to emit a flash like that shown in Figure 9. Similar examples of multipeak flashes in *P. punctulatus* and *P. marginellus* are shown in Figures 12 and 13 of FF-I. Since in such instances there is generally an increase in overall flash intensity, some "new" peaks may represent merely intensification of responses previously too feeble to register. However, Figures 8 and 9, of comparable peak magnitude, show that the additional excitation need not be an amplification artifact. A perhaps analogous instance in the male of *Photuris,* caused by progressively increased stimulus duration, is illustrated in Figures 10, 11, and 12. In Figures 12 and 13 it will be seen that several flashes have intervened, at regular 105-msec. intervals, between the primary responses due to make and break of the current.

Such newly-evoked multipeak responses may maintain their general contour during repeated stimulation so long as stimulus parameters are held constant and stimulus frequency is moderate. It is interesting that the new form may even persist, at least temporarily, after the stimulus is reduced to initial values, indicating lasting facilitation (Figs. 14, 15, 16).

A further suggestion of special cephalic role in stimulation is the fact that voltage, duration or frequency of stimulus, needed to elicit a flash of given magnitude, often increases as the electrode pair is moved progressively forward from abdomen into thorax. When the head is reached, however, parameters may fall to those adequate for stimulating lantern tissue directly.

3. *Central nervous role in response latency*

A. *Cord conduction.* From a large number of measurements on decapitated *Photuris* stimulated at different cord levels, it appears that the typical response delay to anterior thoracic stimulation is in the 110–145-msec. range ("medium latency"). In conjunction with 70–85-msec. latencies to direct lantern stimulation at 22–25° C. (FF-I: Table I and FF-I: Fig. 54), these values suggest cord transit velocities (including junctional delays) of 10–20 cm./sec. for the 7–9-mm. distance. This was confirmed directly in five specimens by making successive anterior thoracic and posterior cord latency measurements on the same individual. There were, however, a few specimens with thoracic latencies of about 90 msec. ("short latency"), indicating cord transit velocities of 40–50 cm./sec., and this also was confirmed by dual-site measurements. Since all determinations were based on numerous measurements and there were apparently no intergrades between the 90 and 110+ msec. latency classes, two excitation modes or pathways are suggested. No bimodal flashes were seen, but since an early flash could blanket a slower response of comparable magnitude (separate flashes are not apparent until paired shocks are 40–50 msec. apart—FF-I) these records do not tell whether short and medium latency responses can occur together.

B. *Brain excitation.* In a search for other indications of dual excitation, intact specimens were stimulated via electrodes in the brain. Some hundreds of such records obtained from five males and three females of *Photuris* seem to fall into two main classes. In one, a single flash of usual intensity occurred with a latency of 120–

150 msec. In the other, the responses involved a very small flash with about a 90-msec. latency and a much brighter and later double flash. These flashes occurred both alone and together in different episodes (Fig. 17). The latency of the large double ("long latency") flash averaged 235 msec. in males and in females seemed to be spread randomly between 500 and 870 msec. after the stimulus (Figs. 21, 22).

The small early element of the response to brain stimulation seems to correspond satisfactorily to the short latency response observed in decapitated photurids, but its characteristic low intensity is unexplained. Occasional atypically large examples can be ascribed to facilitation by a shortly preceding random spontaneous flash (Fig. 18). The 120–150-msec. latency response corresponds to the medium latency response in decapitated specimens. In intact fireflies, therefore, as in decapitated specimens, there appear to be at least two response latencies.

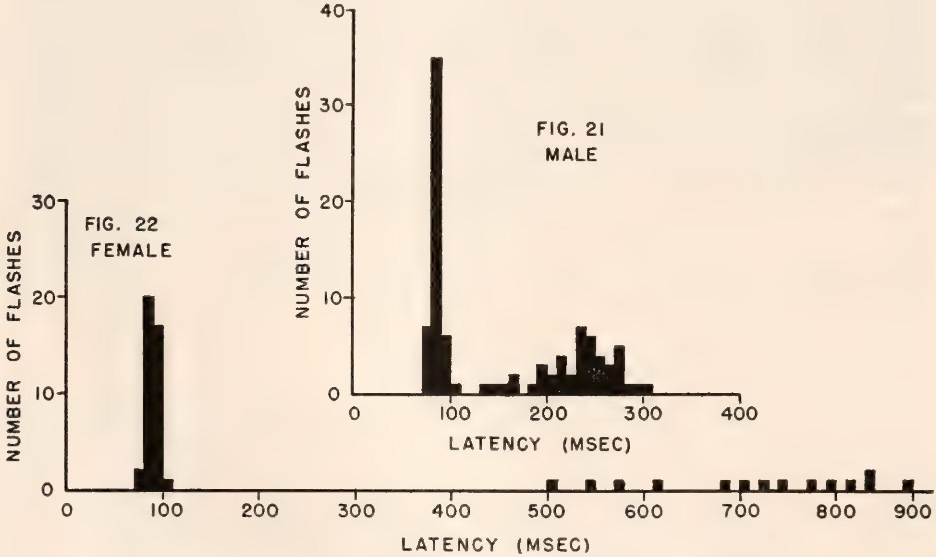


FIGURE 21. Frequency distribution of response latencies to head stimulation in a male of *Photuris.*

FIGURE 22. Frequency distribution of response latencies to head stimulation in a female of *Photuris.*

C. *Central delay.* The long latency response may involve some sort of central delay. This view is favored by the facts that the latency distribution of the late flashes was clearly non-random, at least in the male (Fig. 21). Since the long-latency flashes occurred only in individuals with intact brain-cord connection, they might, alternatively, be ascribed to random endogenous flashing. The relative paucity of flashes in the 0–7- and 100–200-msec. ranges (Fig. 21) could then be due to relative refractoriness of conductor or effector preceding and following the driven flash.

In sum, the roles of brain and cord in excitation seem complex, though some of the response heterogeneity may well reside in lantern tissue. It would clearly be useful to be able to distinguish spontaneous from driven excitations, if they are different, and to detect directly the excitation signal in lantern or cord. To these ends we attempted to record action potentials associated with luminescence.

4. *Photic action potentials*

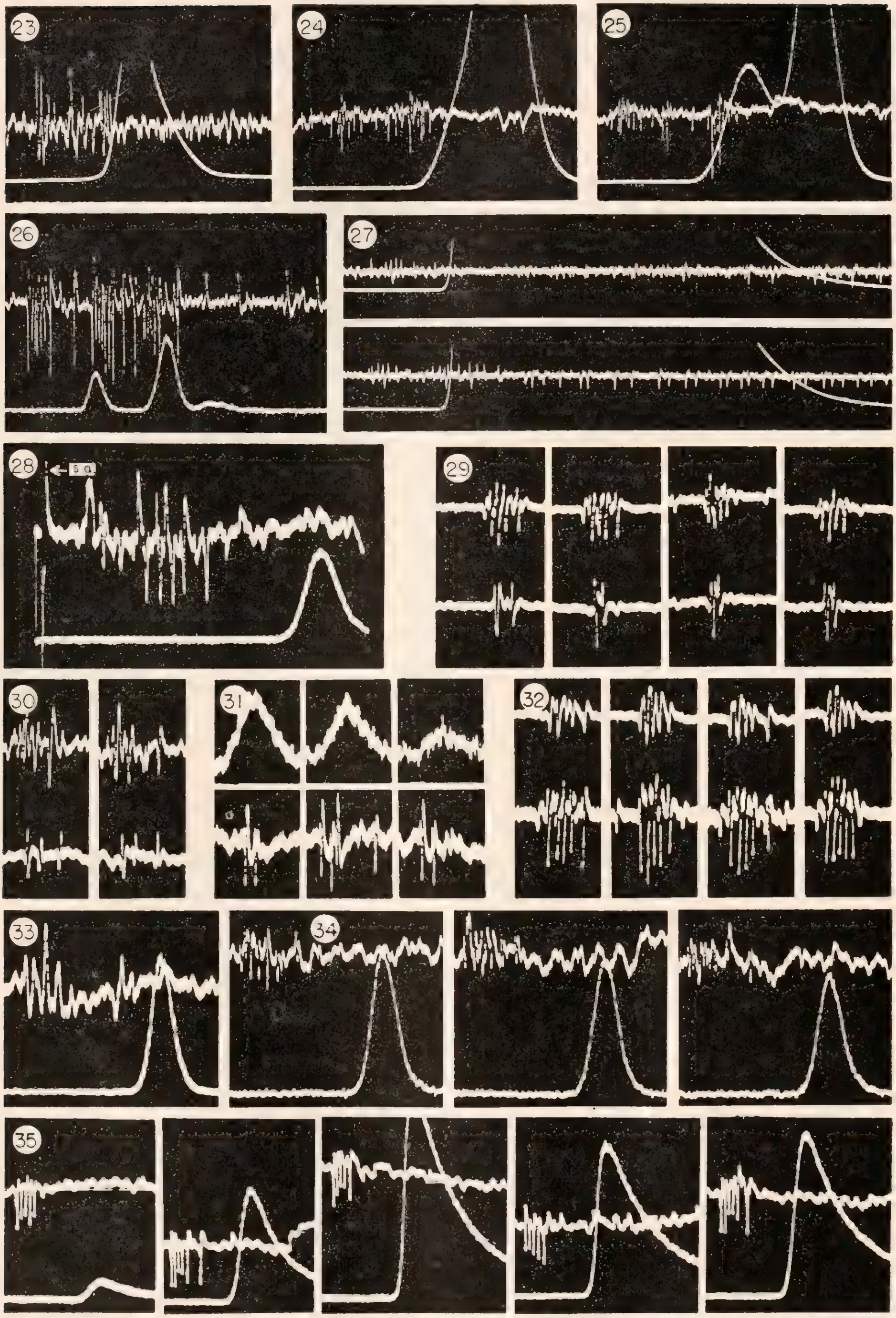
A. *Cord and lantern volleys.* Since *Photuris* is the only firefly among the five species studied that flashes spontaneously with any regularity under laboratory conditions, most of the action potential work was done on this species. Potentials detected in cord lifted free of viscera are much obscured by continuous electrical background which is presumably concerned with musclar activity, but they suffice to demonstrate an unequivocal 1 : 1 relation between small volleys of nerve spikes and succeeding flashes. Records made directly from photogenic tissue, by laying the electrode pair on regions of the lantern surface that have been stripped of cuticle, exhibit less extraneous electrical activity. The volleys thus detected usually correspond closely in number and spacing to those recorded from cord (Fig. 20) and presumably represent the same excitation signals at a more distal point in their pathway—namely, in peripheral nerve. It is often difficult to say exactly when cord volleys begin, but in most instances they seem to start 5–15 msec. earlier than the corresponding lantern volleys as might be expected from the extra junctional and conduction delays that are presumably incurred by the latter.

It is usually not possible to detect much qualitative agreement in spike patterns between cord volleys and corresponding neutral activity in the lantern, although the volleys appear to be of roughly the same duration. There are occasional suggestions that not every cord volley eventuates in a lantern volley (*e.g.,* between first and second episodes of Figure 20) but this may well be due to the difficulty in distinguishing the photic volley from the non-photic background potentials in the cord.

Usually there is a clear correspondence between gross volley structure and gross flash form (contour). Thus, not only are single volleys typically associated with corresponding single flashes (Figs. 19, 33–35, etc.) but double or bi-partite volleys can apparently slow the accretion phase (Fig. 24) of luminescence and, if sufficiently separate in time, lead to double flashes (Fig. 25). Similarly, triple volleys may induce triple flashes (Fig. 26). In other instances the flash seems to be relatively independent of volley duration *per se,* quite similar flashes often resulting whether the volley ends well before luminescence begins or continues even well past the start of the flash (*e.g.,* Fig. 27).

The interval between the first spike of a spontaneous volley and the rise of the resulting flash was 70–90 msec. in most records from *Photuris* (*e.g.,* Figs. 19, 33–35) and 150–175 msec. in the few records obtained from the Iowa *Photinus pyralis* (Fig. 27), values which are close to the latencies for direct electrical stimulation of the lantern in these species (FF-I : Table I).

After electrical stimulation in the head it was possible to record lantern volleys like those detectable during spontaneous flashing (Fig. 28). The delay between stimulus and first spike was about 60 msec., corresponding to a cord transit velocity of about 13 cm./sec. for an 8 mm. path, and the further delay from first spike to flash was 85 msec., which is within the usual latency range for *Photuris.*



FIGURES 23–35.

Such records thus reinforce the idea that luminescence is excited by neural action potentials.

B. *Generality of Signal.* Simultaneous records from spontaneously flashing specimens were made with two pairs of recording electrodes under two conditions: (a) one pair laterally on the segment 6 organ, the other on the center of the segment 7 organ, (b) one pair on the anteromedial part of 6 and the other on a lateral margin of the same segment of the lantern. In a number of such preparations the volleys from different sites were different in spike number although occurring simultaneously or nearly so (Figs. 29, 30). The number of spikes per volley was in fact somewhat variable even in serial records from the same site and could apparently be as small as two or three (Figs. 29, 30, 31, 35). Furthermore, the signals from a given site could sometimes be altered by slight adjustments in electrode position (Fig. 29 vs. Fig. 32; Fig. 33 vs. Fig. 34a). Thus, until able to record action potentials from a known single peripheral nerve together with only the light from the photocytes controlled by that nerve, we cannot identify the minimal or ultimate light-evoking signal. Yet the fact that one sometimes does get similar volleys from widely separated sites (Fig. 32) suggests that a common excitation signal is widely distributed in the lantern.

C. *Spike pattern in relation to flash intensity and flash contour.* In view of the apparent influence of electrode-tissue relations upon action potential pattern and the fact that all our recordings were made from restrained intact animals capable of at least minor body movements, variation between successive volleys from one site might have little intrinsic significance. But flash intensity is itself known to vary somewhat even during an uninterrupted series of normal spontaneous flashes (FF-I) so it is of interest to see if there is any recognizable relation between spike pattern and the intensity of the associated flash.

Comparisons of volleys preceding flashes of nearly identical intensity given by a single firefly indicated that equal flashes are not always preceded by identical spike patterns (*e.g.*, Fig. 34). Conversely, volleys preceding flashes of differing

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FIGURES 23–35. (23) Iowa *Photuris,* female, intact. Spontaneous flash of both organs and lantern potentials from posterior segment. S = 375. (24) Maryland *Photuris,* sex not recorded, intact. Spontaneous flash and lantern potentials. S = 375. (25) As 24. S = 375. (26) As 19. S = 410. (27) Iowa *Photinus pyralis,* male, intact. Spontaneous flash and associated lantern potentials. Two non-consecutive episodes. S = 1470. (28) Woods Hole *Photuris,* male, intact. Lantern potentials and flash in response to stimulation in head with single shock of 2 msec./6 V. Arrow indicates stimulus artifact (S.A.) S = 240. (29) Woods Hole *Photuris,* male, intact. Action potentials from anterior center of sixth segmental organ (top trace) and lateral edge of same segment (bottom trace) during spontaneous flashing. First, 2nd, 10th and 11th episodes in a series. S = 100 for each episode. (30) Woods Hole *Photuris,* male, intact. Action potentials from 6th segment organ (top trace) and 7th segment organ (bottom trace) of same specimen during spontaneous flashing. Fifth and third episodes in a series. S = 70. (31) As 30, except action potentials (sixth segmental organ) are given with accompanying flashes. To save space, flash records are at high gain and are displaced to left with respect to corresponding volleys. Third, 4th and 5th episodes in a series. S = 70. (32) As 29. Episodes 1, 2, 4, and 7 from one series with electrodes having been slightly readjusted from their positions during recording the series of Figure 29. S = 70. (33) Woods Hole *Photuris,* male, intact. Lantern potentials and spontaneous flash. S = 155. (34) As 33. Seventh, 12th and 13th episodes in a series after slight adjustment of electrode position. S = 155. (35) Woods Hole *Photuris,* female, intact. Lantern potentials and spontaneous flashes. Fifth, 13th, 21st, 22nd and 24th episodes in a series of 33. S = 220, 220, 270, 270, 270.

intensities given by a single individual sometimes look quite similar (*e.g.*, Fig. 35, a, c, and d; b and e). However, each main volley in Figure 35 consists of 5 spikes, and careful measurement of the entire series of 33 volleys indicated that the interval between the first and fifth spikes (duration of main volley) varied inversely with peak flash intensity (Fig. 36). A correlation analysis of these data gave a coefficient of −0.48, showing a highly significant association (< 1% for 32 degrees of freedom). Statistically this means that spike frequency by itself can account for about 25% of the modulation of flash intensity.

It had been found previously that flash intensity in a spontaneous series varies inversely with the interval between flashes (FF-I), which should mean a corresponding inverse relation with the interval between volleys. A plot of flash intensity against the interval between the immediately preceding and second preceding volleys does in fact show such an inverse relation (Fig. 37). The correlation coefficient is −0.50 which is significant at the 1% level. Thus, volley frequency by itself also can account for about 25% of the intensity modulation.

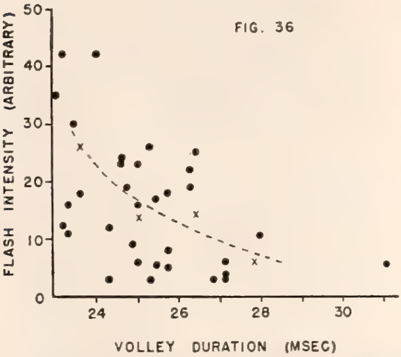


FIG. 36

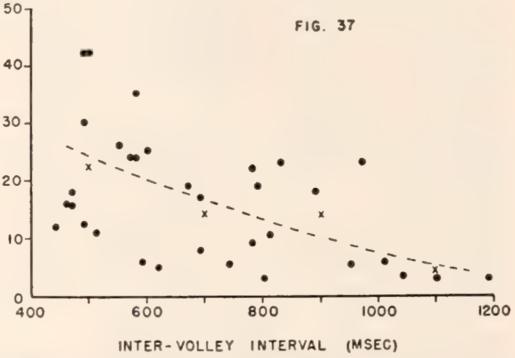


FIG. 37

FIGURE 36. Relation between flash intensity and time span of first five spikes in preceding photic volley. Single Woods Hole *Photuris*, female. Crosses indicate means for about 1.4-msec. groupings.

FIGURE 37. Relation between flash intensity and interval between immediately preceding and second preceding photic volleys. Same Woods Hole *Photuris*, female, as in Figure 36. Crosses indicate means for 200-msec. groupings.

A plot of volley duration for a given episode against the intervolley interval preceding the respective flash indicates association of the two neural variables. The correlation coefficient is 0.53, again highly significant. Spike frequency thus tends to rise as volley frequency rises. The multiple correlation coefficient for flash intensity as influenced by both volley duration and volley frequency is −0.56. This means that because of the high correlation of spike frequency with volley frequency, the combined and approximately equal effects due to these two sources of stimulatory facilitation account for only 31% of the overall influences on flash intensity. Presumably much of the remaining variation is due to non-correspondence of the conductor and effector populations recorded, but there is no reason to exclude the possibility also of modulation at the level of photocyte or neuroeffector junction.

The obvious association of gross volley structure and gross flash form (Figs. 25, 26) suggests that detailed spike distribution might be related to more subtle differences in flash form, such as between symmetrical (Figs. 19, 28, 33, 34, 38, 39) and asymmetrical (Figs. 23, 35) flashes, but in fact even the volleys produced by single individuals of *Photuris* emitting symmetrical flashes show much variation (Figs. 33, 34).

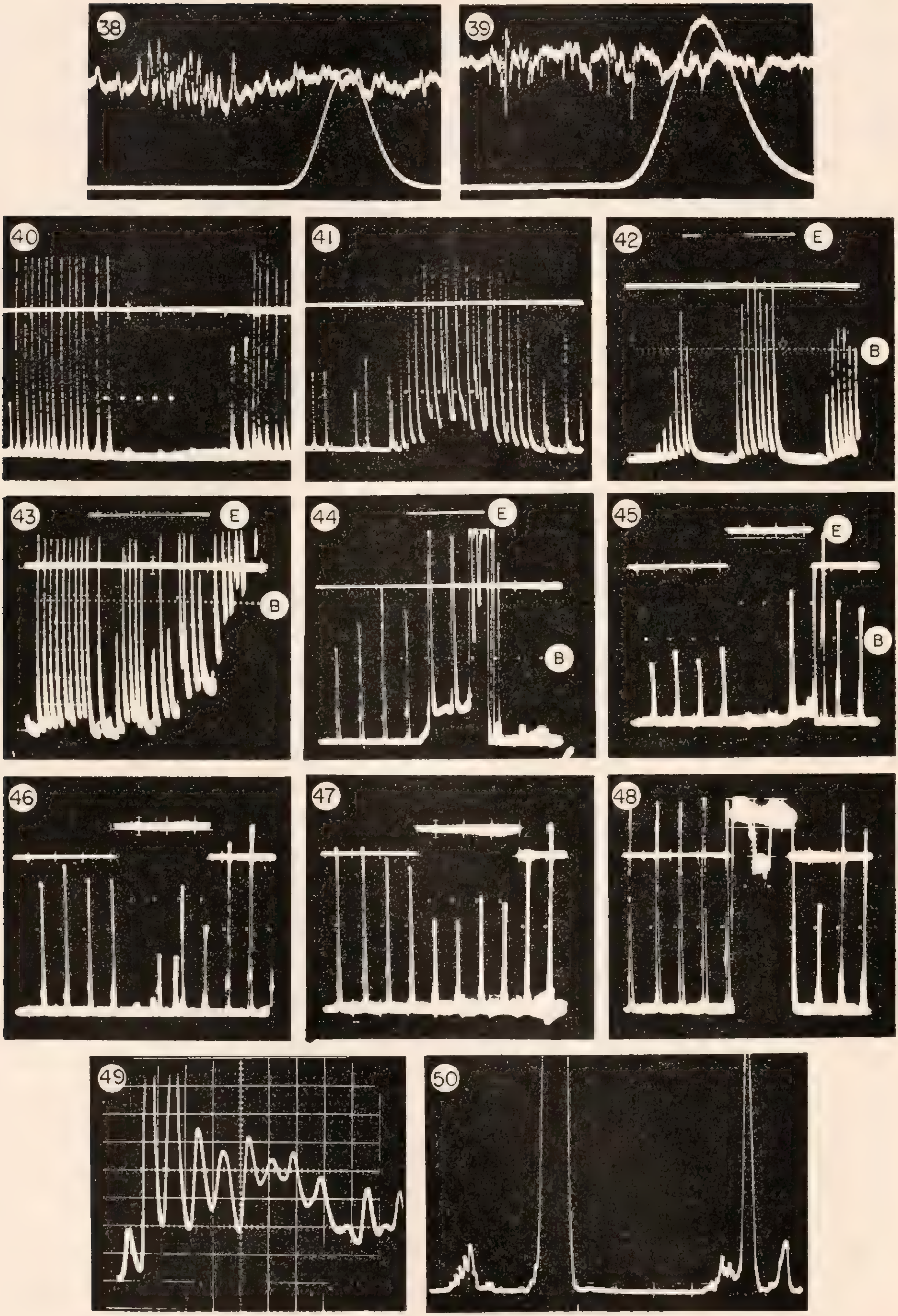
D. *Inter-volley electrical activity.* Isolated single spikes commonly occur throughout the flashing cycle, usually irregularly but sometimes quite regularly (Figs. 19, 27). In our preliminary accounts we suggested that these inter-volley spikes might have a trophic function in sustaining lantern excitability between volleys. Long serial records now have shown, however, that these potentials normally tend to occur in groups lasting 1–1.5 seconds and recurring every 2 to 3.5 seconds, quite independently of the photic volleys. Furthermore, the isolated spikes have a consistently different wave form from spikes in photic volleys. The supposition, therefore, is that they have to do with some other cyclic function, for example, spiracular control. Dr. Albert Carlson (personal communication) has found that the amount of intervolley noise is considerably reduced without affecting the photic volleys if the surface of the lantern is allowed to dry slightly.

5. *Experimental inhibition of luminescence*

The ease with which flashing can be induced when electrodes are inserted in the brain, and the possible express conduction of cephalic stimulation, raised the question of whether specific centers for the excitation of luminescence are present. As a control we therefore tried stimulating an intact animal via electrodes in the eye, which was presumed to be an indifferent cephalic site. To our surprise such stimulation, far from inducing luminescence, sometimes actually suppressed spontaneous flashing. Figure 40 shows an example in which five stimuli one second apart produced an immediate inhibition which lasted for four seconds after eye stimulation ceased. Stimulation by electrodes in the brain of the same animal, on the contrary, enhanced flashing (Fig. 41). Further, stimulation in the eye could also suppress, sometimes after a delay, response of the lantern to stimulation via a second pair of electrodes in the brain (Fig. 42). In such experiments the site of electrode placement in the eye and the relative stimulation frequencies and voltages to eye and brain are apparently quite critical, since partial or complete failure of inhibition by eye stimulation (Figs. 43, 45) or even enhancement of flash intensity (Fig. 44) were observed under some circumstances. Spontaneous flashing, likewise, can either (a) be inhibited essentially completely (Fig. 40), (b) escape from the inhibition after a time even though eye stimulation continues, or (c) not be markedly affected, depending on voltage. An analogous effect, seen with direct current applied to the eye during serial stimulation of the brain, seems to indicate that the response can be quantitatively modified, depending rather critically on relative voltages at the two pairs of electrodes (Figs. 45–48). Details of this inhibition will be considered in another communication.

6. *Effect of eserine*

A number of agents (spider venom, hypoxia, cyanide, ether, etc.) are known to upset effector co-ordination and bring about asynchronous lighting of minute



FIGURES 38–50.

areas of the lantern ("scintillation"). We will consider the morphological nature of the small luminous units elsewhere: for present purposes it suffices to say that they comprise both single photocytes and small aggregations. We found that 10^{-3} and 10^{-4} M eserine perfused through the abdomen also induces scintillation. The frequency of firing (13–17/sec.—Fig. 49) was close to the limit of 1:1 response to train stimulation (FF-I). In some preparations the eserine effect was particularly dramatic in that the bouts of sparkling alternated regularly with intervals of near darkness (Fig. 50). Since scintillation is a nonspecific response there is no compelling reason to homologize the action of eserine in fireflies with that in better known systems but the relatively sudden onset of the paroxysms of luminescence and their equally sudden quenching bear a remarkable resemblance, for example, to the activity-block cycles of post-synaptic elements of eserinized cockroach cercal ganglia (Roeder *et al.*, 1947).

The drug was ineffective in inducing scintillation in a deganglionated light organ, but since the photogenic tissue is well insulated from the hemocoel by the "reflector" layer of the lantern it is not certain that peripheral junctions were actually exposed.

Intensive efforts to record potentials from lanterns of eserinized fireflies both intact and decapitated did not yield any information about unit activity.

7. *Excitatory state*

Specimens often show very low threshold for electrically-induced flashing when first mounted with electrodes, the threshold then rapidly rising to a higher level which remains stable for an hour or more. Whether the initial condition is one of hyperexcitability induced by handling or the later state one of adaptation is unknown but for reproducible records we customarily waited about 15 minutes after first mounting the specimen.

Fireflies are occasionally refractory. This refractoriness is manifested by inordinately high electrical thresholds, dim induced luminescence, lack of spontaneous flashing and lethargic behavior. It seems not clearly correlated with time of day, but this is not necessarily conclusive because of the irregular illumination regimen of most specimens over their usual several days of laboratory life. Full responsiveness can usually be restored by handling or other irritation. Scratching the head with a needle, particularly if the cuticle is broken, is usually effective, as is

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FIGURES 38–50. (38) Maryland *Photuris*, probably male, intact. Lantern potentials and spontaneous flash. S = 300. (39) Maryland *Photuris*, probably male, lantern potentials and spontaneous flash. S = 300. (40) Woods Hole *Photuris*, male, intact. Spontaneous flashing. The five dots denote stimuli to eye, 15 msec./10 V., 1/sec. S = 18 sec. (41) As 40. Stimulator trace: train of six shocks to brain of 15 msec./10 V., 1/sec. S = 18 sec. (42) Woods Hole *Photuris*, male, intact. Top stimulator trace (E), two trains of stimuli to eye, 4 msec./10 V., 30/sec. Lower stimulator trace (B), stimuli to brain of 2 msec./12 V., 2/sec. S = 21 sec. (43) As 42. Top trace (E): train of stimuli to eye of 4 msec./10 V., 10/sec. Lower trace (B): brain stimulation at 4 msec./7 V., 2/sec. S = 21 sec. (44) Woods Hole *Photuris*, female, intact. Top trace (E): stimuli to eye of 5 msec./15 V., 40/sec. Lower trace (B): brain stimuli of 3 msec./14 V., 1/2 sec. S = 21 sec. (45–48) As 44 except eye stimuli are, respectively, 7 V. DC, 6 V. DC, 5 V. DC and 8 V. DC. S = 21 sec. (49) Iowa *Photuris*, sex not recorded; 10^{-3} M eserine in hemocoel. Flash frequency about 13/sec. S = 1150. (50) Iowa *Photinus pyralis*, male, luminescence of isolated organ after hemocoel injection of 10^{-4} M eserine. S = 5 sec.

chasing the animal around on the table top for a minute or so before it is fastened down for experimentation. It is significant that any arousing has to be done before decapitation or cord section, as if the state of the central nervous system determines the responsiveness of the whole excitation pathway.

In addition to involving a transition from a motionless individual, standing with bowed head, to an actively walking firefly with antennae waving, the "arousal syndrome" has interesting luminescent manifestations. As the animal becomes disturbed the originally dark lantern begins to show dim irregular local flecks or blotches of light which grow progressively. Eventually the whole lantern may blush dimly and then finally emit a bright flash followed by total extinction. After this, normal spontaneous flashing can occur.

DISCUSSION

Both voluntary and electrically-induced flashes are invariably preceded by characteristic volleys of action potentials which can be detected in the cord and in peripheral nerve within lantern tissue. This evidence, along with Hanson's (1962) experiments on the gross conduction pathways of the lantern, demonstrates directly for the first time that the control of bioluminescence can be along conventional neuroeffector lines.[3] We have also observed a variety of spontaneous and induced luminescent phenomena which, in spite of the limitations of our extracellular recordings, the present unavailability of unit conductor-effector preparations, and our ignorance of modulating potentialities of the effector tissue itself, suggest the following further details of central nervous involvement in flash control.

1. *The excitation signal*

The rhythmicity of spontaneous flashing, its cessation upon decapitation, and the low threshold to cephalic stimulation point to the brain as the normal trigger for flashing and the site of a pacemaker of remarkable regularity. Volleys clean enough for detailed analysis could not be recorded even from the posterior part of the cord but it is a reasonable postulate that both spontaneous and electrically induced flashing depend on central generation and propagation of volleys similar to those recorded from peripheral nerve. This signal is specific in its close sequential association with flashing and with no other visible activity. Its gross similarity in cord and lantern and its nearly simultaneous arrival in different parts of the luminous tissue point to a general excitation of the lantern.

The statistically demonstrable modulation of flash intensity by the photic volley shows effector facilitation by the action potential spikes, and also by volley frequency. There is often a rough correspondence between spike groupings in multiple volleys and the general form of the corresponding flash but the finer details of single flash contour are apparently not associated directly with specific spike patterns within the volley. Rather it seems likely that the time course of luminescence during spontaneous flashes—for example whether the contour is symmetrical or the rise more rapid than decay—depends primarily on the response characteristics of the effector cells or upon the topographic distribution of excitation pathways (Buck, 1955).

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[3] In some Japanese and Korean fireflies light is not emitted as brief flashes separated by complete darkness, but as a long-lasting glow fluctuating slowly between bright and dim. Hasama, in a series of studies on such species (*e.g.,* Hasama 1939, 1942), figures monophasic "action potentials" detected with paired non-polarizable wick electrodes, one resting on the external cuticle of a non-luminous segment, the other on a luminous segment (said to be more negative). These string galvanometer records, which consist of rhythmic sawtooths with a period of 5–6 seconds, were said to correspond "fast ganz" to the frequency of light emission. The latter was not recorded but was said to be 15–20/sec. The potentials were reported not to correspond to the ventilatory rhythm but were found to be temporarily enhanced in magnitude, and slightly in frequency, by oxygen. The relation between Hasama's potentials and ours is uncertain, but it seems clear that the Japanese worker cannot have been dealing with conventional nervous action potentials.

2. *Latency, central delay and conduction velocity*

Measurements on intact and decapitated specimens of *Photuris* stimulated at the anterior end of the cord indicate three head-to-lantern latency classes. The head-dependent, long-delayed (200+ msec.) flash is ascribed tentatively to central after-discharge. The short (ca. 90 msec.) and medium (ca. 120 msec.) latencies might reflect excitation via one or the other of two pathways conducting at different velocities, the variability in excitation being perhaps related to the crudeness of the stimulating electrodes and their variable placement. Alternatively, the longer latency might be due to delay in brain or cord. Unfortunately our action potential records are of little help in choosing between dual pathways and central delay, because only one stimulus-to-volley latency class, corresponding to the slower conduction velocity, is present in the few records that show artifact, volley and flash together.

Behavioral data from *Photinus pyralis* demonstrate another ambiguity of central nervous latency. In the nuptial signaling of this species the male cruises about, flashing every 5.8 seconds (at 25°). The perched female does not flash except in response to a male that flies within 20 feet or so of her, in which case she replies to his flash after an interval of about 2 seconds. Now, the reaction time of the female is fairly precise—in fact this is the crucial cue that enables the male to distinguish her flash from those of other males (Buck, 1937b). Hence the 2000 msec. could very properly be called the latency of her presumably reflex response to ocular stimulation, even though it is obviously quite a different kind of latency from the 200–300 msec. that would presumably be measured for flashing in response to strong electrical stimulation of the eye in this species.[4] (It is interesting, incidentally, that the female's flash is triggered by a stimulus (light) that would be strongly inhibitory were it somewhat more intense.)

3. *Types of endogenous rhythm*

Fireflies exhibit several types of rhythmic or repetitively patterned luminescence.

Insofar as the potentialities of the central nervous system in exciting such flashing are concerned there is probably no intrinsic reason for excluding the high frequency flashing that occurs only during or shortly after vigorous stimulation (Fig. 1, 4–7) or the apparently ganglion-dependent pulsing luminescence of eserinized fireflies (Figs. 49, 50). Similarly it seems very likely that the regular multipeak flashes induced electrically (Figs. 9, 15) involve much the same excitation process and sequence as compound flashes that occur without any obvious external stimulation (Fig. 2). There is, however, some conceptual simplification in restricting the consideration of rhythm to "normal" or "spontaneous" manifestations. These include the repetitive flashing during flight, the production of multipeak flashes and the inherent diurnal activity rhythm.

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[4] Estimated on basis of the 150-msec. direct lantern latency (FF-I, Table I) plus 100 msec. for cord transit.

The head-dependent, highly species-characteristic and often intricate flashing patterns of flying males of many New World lampyrid fireflies illustrate the precision of programming attained by the central nervous system. These signals are not only regularly repeated usually at intervals of several seconds, but may display a remarkable constancy of timing and relative intensities of sub-flashes or peaks within each flashing episode (McDermott, 1914; McDermott and Buck, 1959). Previous studies have also shown that this spontaneous rhythm is temperature-dependent to a degree similar to those of insect neuromuscular activities such as ventilation (Snyder and Snyder, 1920; Buck, 1937b).

Compound flashes themselves illustrate another type of repetitive luminescence that is possibly dependent on the central nervous system. Three mechanisms seem possible: (1) sub-peaks could be due to synchronous repetitive firing of photocytes in response to repetitive volleys from the central nervous system (*e.g.*, Fig. 26). The fact that similar flashes can be elicited from isolated lanterns indicates that excitation need not involve more than one or two ganglia of the cord. (2) The sub-peaks could represent responses of photocyte populations differing in latency. The oscilloscope record would look the same whether these populations were spatially separate or intermingled, and because of the high frequency of the repetitive flashing in each episode it would usually not be possible to distinguish visually between these alternatives. However, on rare occasions it appears that the two segmental organs or separate areas of one segment may flash slightly out of phase with each other. (3) The sub-flashes could reflect a conductional pattern leading to asynchronous excitation of different photocyte populations (Buck, 1955).

The 24-hour cycle of flashing activity is little known but is presumably controlled by a still different pacemaker mechanism from the above. This, in contrast to numerous other biological clocks, seems to vary with mid-range temperature (Mather, 1947). Curiously, also, this long-period cycling is inhibited by light intensities both above and below a relatively narrow range of ambient values (Buck, 1937a). Hence, like the modifications in the species flash pattern that occur during the mating signals, the pacemaker is responsive to sensory input.

4. *Excitatory state*

Many qualitative and quantitative characteristics of flashing are reasonably stable over at least limited periods and vary predictably and directly with stimulus parameters (FF-I). There is also much evidence that the whole level of excitability may become high, as in the initial low threshold period and in post-stimulatory luminescence, or low, as in the examples of refractoriness described. The involvement of the central nervous system in some of these phenomena is indicated by the necessity for an intact head-cord connection during arousal and in the restriction of delayed post-stimulatory flashing to intact specimens. Further, Carlson (1961) found that even such an ostensibly involuntary or automatic response as the "pseudoflash," evoked in hypoxic fireflies by sudden readmission of oxygen, is reduced during refractory periods of the diurnal cycle unless the animal is aroused before subjection to hypoxia. It is interesting, in this connection, that the pseudoflash can only be induced at a stage of hypoxia so severe that the peripheral nerves are electrically silent. Hence it appears that the necessary degree of peripheral facilitation depends on prior central activity. In regard to inhibition, also, our experiments with electrical stimulation via the eye at least suggest the possibility of an interplay between stimulatory and inhibitory centers in the brain.

The question of excitation in the post-ganglionic portion of the excitation pathway will be considered in the third paper of this series.

Summary

1. The central nervous system is shown to be involved in (a) normal spontaneous flashing, both single and multiple, (b) some types of post-stimulatory flashing and scintillation, (c) comatose behavior and refractoriness to stimulation.

2. Two and probably three latencies in response to head and anterior cord stimulation exist. At present it is not possible to distinguish between cord pathways (conducting at ca. 15 and 50 cm./sec.) and central delay as possible causes of these latency differences.

3. From posterior cord and from lantern surface it is possible to record small and characteristic volleys of action potentials associated 1:1 with spontaneous flashing and involving latencies comparable with those previously found for electrical stimulation. Multiple volleys may invoke multiple flashes. Flash intensity increases with both volley frequency and spike frequency but there is apparently not a close relation between volley structure and flash contour.

4. Electrical stimulation in the eye can either inhibit or enhance flashing, depending on relative intensities of brain and eye stimulation.

5. In preparations including ganglia the anti-cholinesterase eserine can induce both asynchronous activation of small units and a recurrent alternating large scale activation and block of luminescence.

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CONTROL OF FLASHING IN FIREFLIES.
III. PERIPHERAL EXCITATION

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In preceding papers (Buck and Case, 1961; Case and Buck, 1963; hereinafter designated "FF-I" and "FF-II") we described responses of five species of lampyrid firefly to electrical stimulation and explored the role of the central nervous system in excitation and modulation of luminescence. Some of these observations also bear on properties of peripheral parts of the excitation pathway. For example, we found that both normal spontaneous flashing and that triggered by electrical stimulation of the brain are excited by characteristic volleys of action potentials detectable in the cord and also throughout lantern tissue, presumably in peripheral nerve (FF-II). Likewise, the finding that only about a third of the variation in flash intensity is demonstrably dependent on variation in associated neural signals (FF-II) suggests the possibility of modulation at neuroeffector junction or photocyte level.

Among other indications that the eventual production of a flash involves more than an electrical signal and a conduction pathway direct to the photocyte are the facts that photic volleys can be recorded at a stage of hypoxia that prevents flashing (Carlson, 1961) and that flash intensity can be influenced by "priming" stimulation and by volley frequency, both of which involve effects persisting far longer than one would expect of purely neural facilitation (FF-I, II).

One of our most provocative findings is that response latency is long. In *Photuris* the delay between time of stimulation in the head and the resulting flash is usually 90–120 milliseconds (msec.) at 20–25° C. Even with the electrode pair directly in photogenic tissue, latency is between 65 and 85 msec. and up to 50 msec. can elapse between the last spike of the normal photic volley and the start of luminescence. It thus takes more than twice as long for excitation to pass from cord to photocyte, a distance that cannot exceed about 2 mm., as to traverse the entire 8–10-mm. length of the cord. The cord-to-photocyte latency undoubtedly includes not only conduction delay in peripheral nerve fibers but delay in some sort of neuroeffector junction.

The only available measurement bearing directly on peripheral conduction velocity is the 5–15-msec. delay between the time a spontaneous photic volley is detected in the posterior cord, dorsal to the lantern, and the time of its arrival on the ventral surface of the photogenic tissue (FF-II). The point in the peripheral innervation from which such potentials are recorded is not known exactly, but it probably lies near to the distal nerve endings, hence indicating that only

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[1] Aided by N.I.H. Grant B-1890.

a few milliseconds are occupied in actual conduction with the lantern. This deduction is also supported by the fact that the volley-flash delay in spontaneous flashing is so nearly the same as the stimulus-flash latency in flashing induced by direct stimulation of the lantern (FF-I, II). Further, pair-shock experiments demonstrate that detectable augmentation of response can be produced by a test shock occurring at any time from a few milliseconds after the conditioning shock to a few milliseconds before the flash begins (FF-I). However, in view of the lack of direct information about size and pathway of the finer fibers and the nature of their endings, it is not excluded that conductional delay is a major fraction of overall peripheral latency. The present paper is accordingly devoted to trying to define more precisely the sequence and path of peripheral excitation.

Materials and Methods

The lantern of the adult firefly consists of a flat luminous organ just inside the ventrical cuticle of both of abdominal segments 6 and 7. They are innervated from ganglia 4–6 of the ventral cord. The larval organs are a pair of small discs located ventrolaterally in the 8th abdominal segment. Methods for stimulating light production electrically and for recording the light emission are given in our two preceding papers. Unless otherwise noted the responses described in this paper were evoked via an electrode pair of 0.005-inch bare silver wires placed directly in lantern tissue, 1–2 mm. apart. The stimulus source was a Grass S-4 stimulator connected through a stimulus isolation unit. The nominal stimulus voltages used in the various experiments have comparative value only, since tissue resistance was not measured and we were in any case dealing with a multicellular response system in which overall potential difference gives little indication of actual current flux per cell. Further details concerning species of firefly studied and methodology are given in FF-I and FF-II.

Results

1. *Peripheral excitation pattern*

The normal spontaneous firefly flash is so brief and brilliant that it is extremely difficult to make out local details, but excitation usually appears to be simultaneous and uniform over the entire area of the lantern. This is true also for the vast majority of driven flashes. Occasionally, however, spontaneous or induced flashes may appear only in certain parts of the lantern or may involve slight asynchrony between different areas of photogenic tissue (see also FF-II). If only part of the lantern is active it is apt to be the central portion of the sixth sternital organ, the area of luminescence often increasing with successive flashes. However, lateral regions may upon occasion flash without the central portion. Many of these local responses can be interpreted in terms of the gross distribution of the peripheral nerves (Hanson, 1962).

In photurids that were emitting mixtures of single, double, and triple spontaneous flashes a 0.005-in. thick brass sheet, pierced by three 200-μ holes about 800 μ apart, was applied closely to the lantern in such a way that one hole lay over the center of the anterior edge of the segment 6 organ, one lay over the posterior edge of the same segment, and the third lay over the posterior edge of the seventh segmental organ. By covering the holes two at a time and recording the light passing through the third it was shown that each of the three widely separated areas of the lantern, each amounting to less than 1/50 of the total area, was able to produce single, double, and triple flashes. Though quite variable in contour the multiple flashes were comparable in peak timing to those recorded from the whole unmasked lantern (FF-II, Fig. 2). This suggests that usually the sequence of spontaneous excitation of the lantern is similar in all areas. The same conclusion was indicated by the fact that similar action potentials can be recorded from different sites in the lantern (*e.g.*, FF-II, Fig. 32).

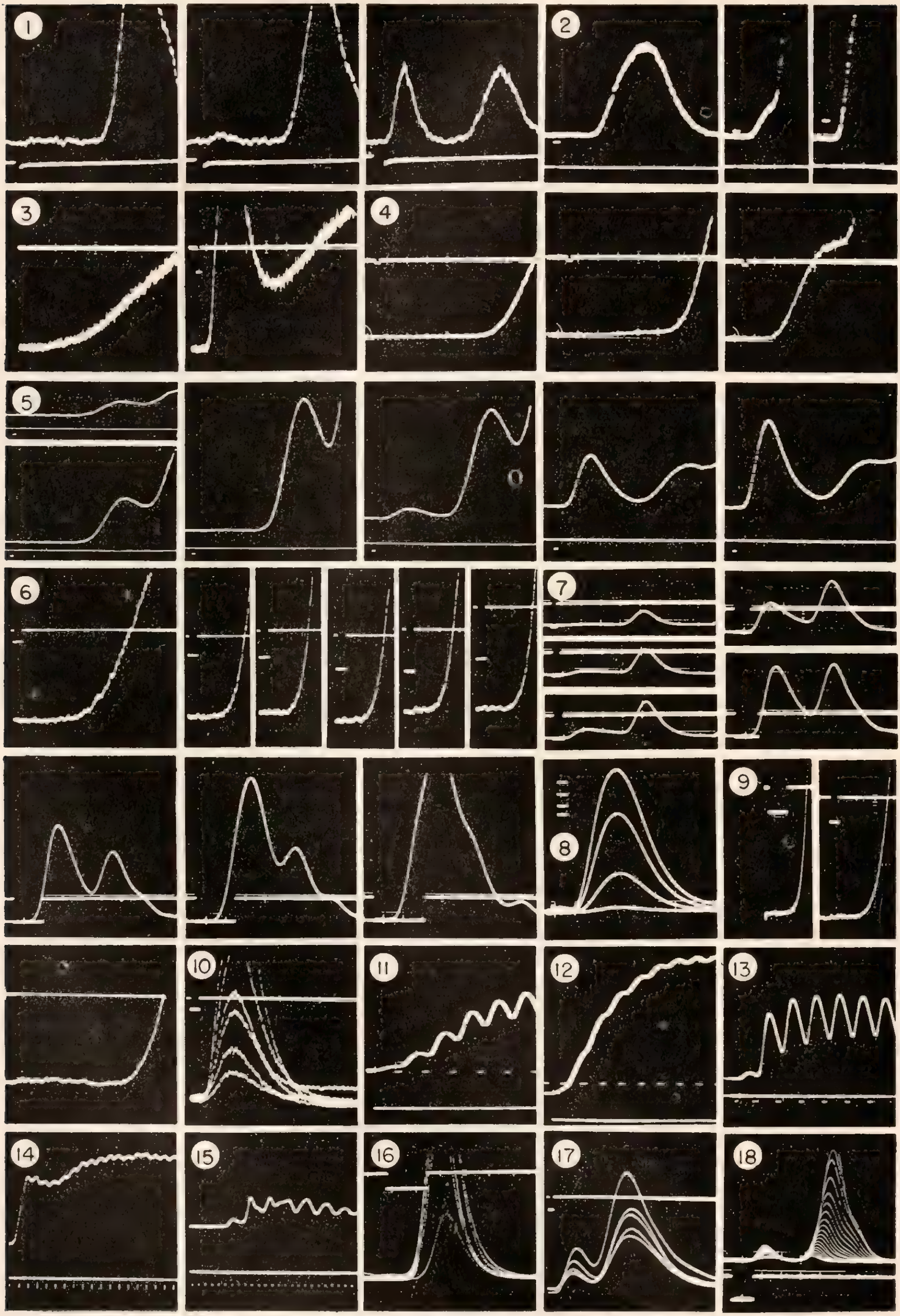
2. *Short latency response of adult*

Adult fireflies at room temperature ordinarily respond to shocks of only a few volts intensity and a few msec. duration, particularly if stimulated directly in the lantern. When stimulus strength reaches quite high values a new short latency response often appears instead of or in addition to the usual flash. This new response we call a "quick flash" to distinguish it from the usual longer latency "slow flash" described in the first two papers of this series.

Figures 1, 3, 7 and 9 illustrate quick flashes induced in various species by increasing stimulus duration, and Figures 2, 4, 5 and 6 the same for increasing voltage. Figure 5 shows the development of the quick flash in a species with multi-peak slow flash (*P. punctulatus*). The apparent merging of slow and quick flashes seen in some figures (*e.g.*, 2, 4, 6), as contrasted with records in which the responses remain distinct (Figs. 1, 3, 5, 7), can be brought about by excessive quick flash duration due either to high amplification of the light trace or strong stimulation.

As we shall show, the interrelations of slow and quick flash are quite complex. In a general sort of way, however, the slow flash is the characteristic response to weak or moderate stimulation, the quick flash is generally dominant with very strong stimulation, and both responses may occur together at intermediate stimulus intensities. Both types of flash may apparently involve the total lantern surface; however, because of the short interval between quick and slow flashes and because of the diffusion of light in all directions through the lantern tissue this is a point about which it is very difficult to be certain. For the same reasons we have as yet no certain answer as to whether both quick and slow flashes can be produced in sequence by the same cell. There were one or two instances in which quick and slow flashes were apparently confined to different segmental organs, but this is not necssarily indicative of a separate photocyte population for each type of response since stimulus current density may have been subliminal in one region and not in the other or one response may have been fatigued (see below).

A. *Threshold.* Stimulus duration and voltage thresholds for quick flash induction vary somewhat from individual to individual and species to species but often appear to be quite sharp in a given individual (Figs. 1–4). However, if levels are graded sufficiently closely it is usually possible to show that the quick flash builds progressively with increasing vigor of stimulation (Figs. 5–7) and that the effect is reversible (Fig. 9). Threshold not uncommonly falls with repeated stimulation, perhaps indicating facilitation. For example, whereas the quick flash might appear first at a nominal 20 msec./150 volts (in *Photuris*), only



FIGURES 1–18.

10 msec./60 V. might be sufficient after several successive trial series. Nevertheless it is sometimes possible to obtain reasonably conventional strength-duration data from individuals (*e.g.,* Fig. 19).

Quick flash thresholds for the small species, *Photinus marginellus* and *P. consanguineus,* are generally much lower than for *Photuris* and the large species of *Photinus.* This and other evidence discussed below indicate that threshold is importantly influenced by tissue mass and electrode placement. For this reason no reliance can be placed on our absolute threshold values. Since, however, the conditions of stimulation for slow and quick flash in the same species should be at least roughly comparable, the values of 10 msec. for chronaxie and 30 volts for rheobase for the quick flash of *Photuris* (Fig. 19) probably form a valid contrast with the corresponding slow flash values of 3.9 msec. and 2.1 volts (FF-I).

Stimulus strength has to be increased drastically in order to elicit slow flashes at low temperatures, and no slow flashes have been detected in *Photuris* below about 8° C. (FF-I). In contrast, excitation threshold of the quick flash seems nearly or quite independent of temperature, and responses have been elicited down to at least 3.5°, a temperature at which slow-flashing is probably impossible (*i.e.,* latency is infinite: Fig. 23; FF-I, Fig. 45).

B. *Latency.* The most striking and characteristic difference between slow and quick flash is in latency. In *Photuris* (Figs. 1, 7), *Photinus pyralis* (Fig. 17), *P. punctulatus* (Fig. 5) and possibly in *P. consanguineus* (Fig. 24) there is

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FIGURES 1–18. (In these and other oscillographs the time scale is from left to right and is given as S = entire width of picture in msec. Figures identified "As X" refer to the same individual as that of Figure X. Some figures slightly retouched.) (1) Woods Hole *Photuris,* male, isolated 6th segment organ. Three successive responses to shocks of 5 msec./150 V., 7/150 and 10/150. S = 150. (2) *Photinus marginellus,* male, isolated lantern. Three successive responses to shocks of 10 msec./7 V., 10/10 and 10/12.5. S = 875, 225, 225. (3) *Photinus consanguineus,* male, intact. Two successive responses to 5 msec./150 V. and 10/150. S = 440. (4) As 3. Three successive responses to 5 msec./20 V., 5/25 and 5/30. S = 175. (5) *Photinus punctulatus,* male, isolated 6th segment organ. Six successive responses to 1 msec./20 V., 1/40, 1/60, 1/80, 1/100 and 1/120. S = 175. (6) *Photinus marginellus,* male, decapitated. Six successive responses to 5 msec./40 V., 5/60, 5/80, 5/100, 5/120 and 5/150. S = 85, 30, 30, 30, 30, 30. (7) Iowa *Photuris,* male, isolated abdomen. Eight successive responses to duration series of 5, 7.5, 10, 15, 20, 30, 40 and 50 msec., voltage probably 150. S = 175. (8) *Photinus consanguineus,* male. Four superimposed successive responses to 10 msec./60 V., 10/90, 10/120 and 10/150. Stimulus artifacts above Figure number. S = 175. (9) Iowa *Photinus pyralis,* male. Three successive responses to 10 msec./150 V., 5/150 and 1/150. S = 50, 50, 92. (10) Iowa *Photinus pyralis,* male, decapitated. Six superimposed successive responses to 20 msec./150 V. S = 430. (11) Iowa *Photinus pyralis,* male. Response to train of 10 msec./150 V. shocks at 7/sec. S = 850. (12) As 11, but 10/sec. S = 850. (13) *Photinus punctulatus,* male, isolated 6th segment organ. Response to train of 2 msec./70 V. shocks at 30/sec. S = 250. (14) *Photinus punctulatus,* female, isolated organ. Response to train of 5 msec./50 V. shocks at 20/sec. S = 950. (15) *Photinus punctulatus,* male, isolated 6th segment organ. Response to train of 1 msec./15 V. shocks at 30/sec. S = 850. (16) Woods Hole *Photuris,* female, recording from 7th segmental organ in otherwise intact animal from which 6th segment organ had been removed. Superimposed responses to 20 successive shocks of 30/msec./150 V. at 2/sec. S = 145. (17) Iowa *Photinus pyralis,* male, decapitated. Five successive responses to shocks of 20 msec./150 V. S = 860. (18) Iowa *Photuris,* male, isolated abdomen. Twenty successive responses to 20 msec./150 V. at 2/sec., superimposed. In this series the quick flash augmented somewhat with repeated stimulation while the slow flash decreased progressively in intensity. S = 175.

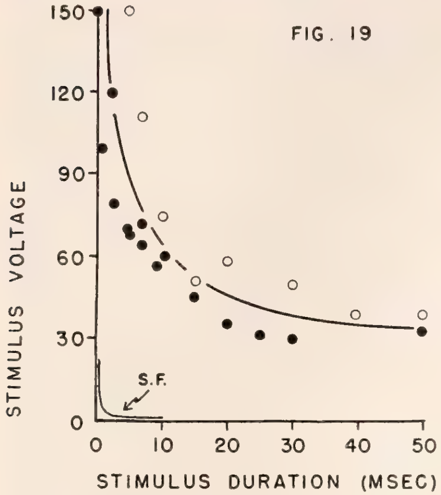


FIG. 19

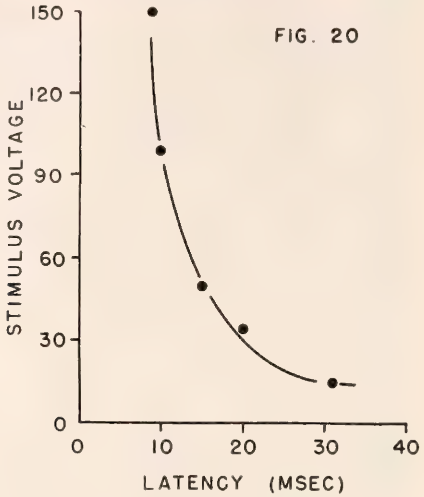


FIG. 20

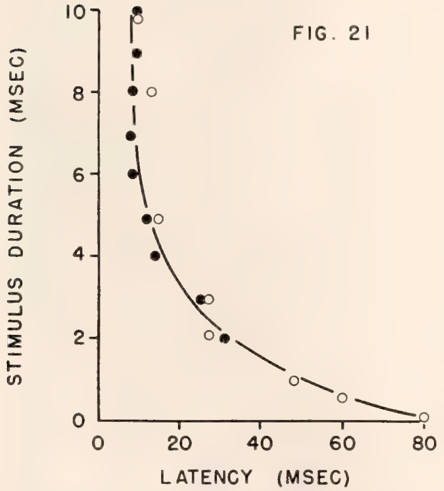


FIG. 21

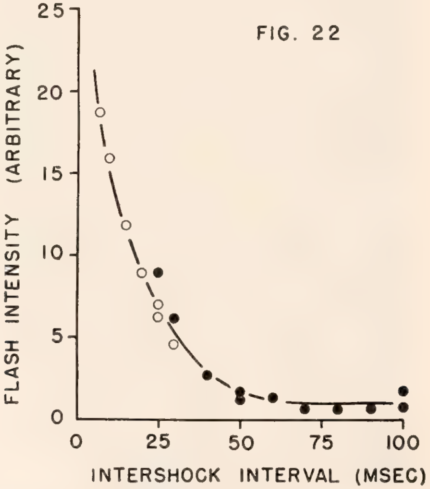


FIG. 22

FIGURE 19. Strength-duration relations for quick flash threshold in two representative individuals. Solid circles, Woods Hole *Photuris,* male. Open circles, Maryland *Photinus pyralis,* male. "SF" indicates corresponding curve for slow flash of *Photuris* (from FF-I).

FIGURE 20. Relation between quick flash latency and voltage of stimulus. *Photinus consanguineus,* male.

FIGURE 21. Relation between quick flash latency and duration of stimulus. Solid circles, *Photinus consanguineus,* male. Open circles, Iowa *Photinus pyralis,* male.

FIGURE 22. Facilitation of quick flash by paired shocks. *Photinus punctulatus,* male. Solid circles, first run. Open circles, repeat run.

usually no question of the independence of the two phenomena, although the actual latency values may appear to vary somewhat with flash magnitude because of uncertainty in deciding the exact initiation point (*e.g.*, Fig. 4b). For this reason, also, latency may appear to be shorter with higher stimulus voltage (Fig. 8) or duration (Figs. 3, 7, 9). However, in the high intensity range of stimulation, when the lantern is well facilitated, latency often appears to be independent of flash amplitude (Figure 6). Thus with incremental changes in voltage (Fig. 20) and duration (Fig. 21), latency tends to approach a limiting minimum value. From such measurements it was possible to compile the quick flash latency estimates given in Table I.

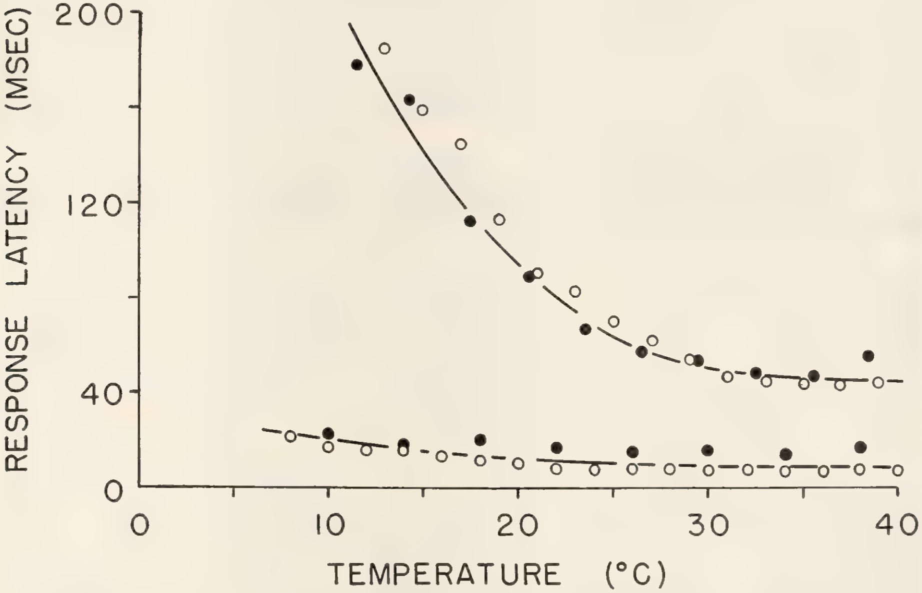
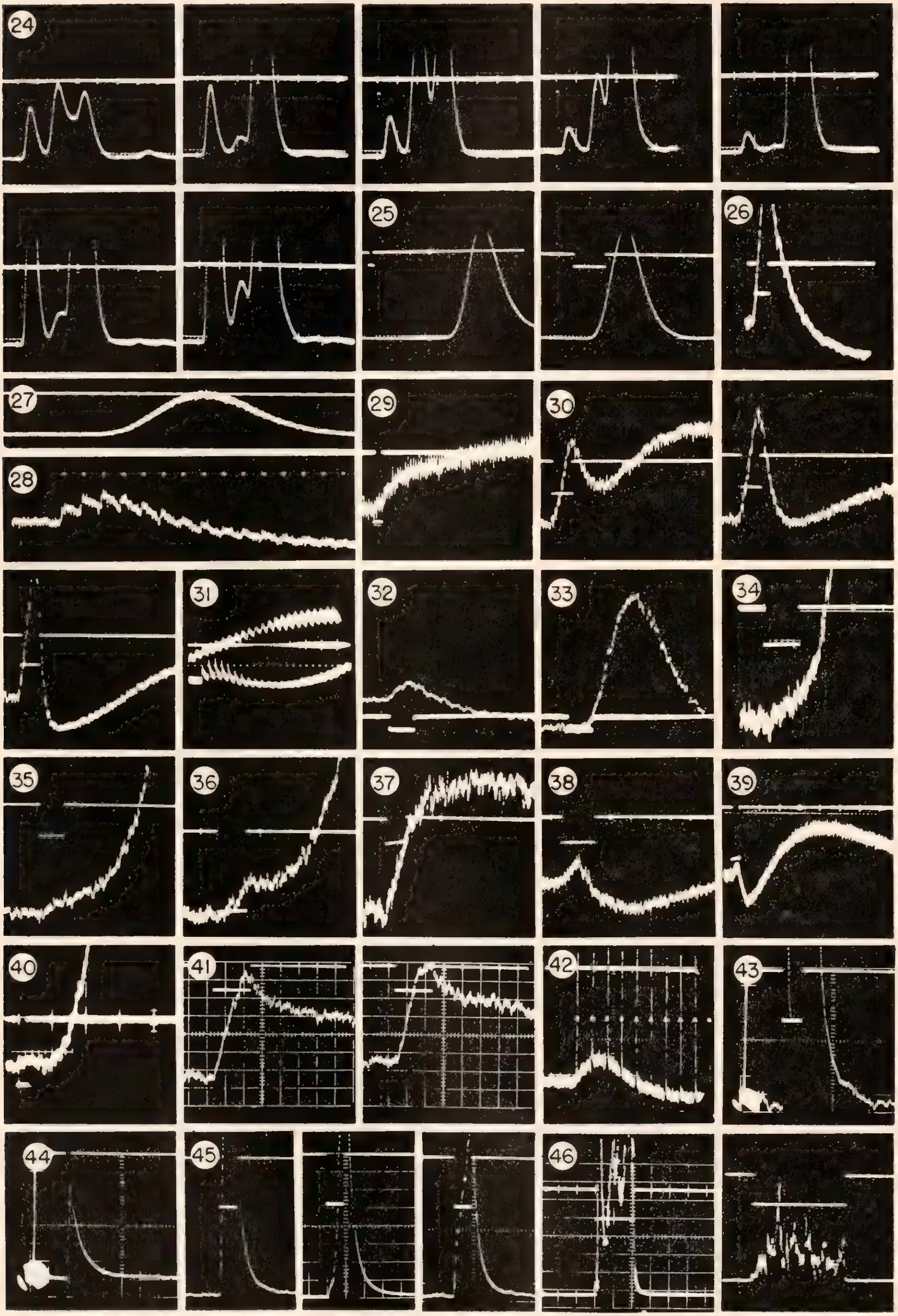


FIGURE 23. Latency in relation to temperature. Solid circles, *Photinus punctulatus*. Open circles, Woods Hole *Photuris*. Quick flash data: means for 113 measurements from 6 males of *P. punctulatus* and 37 measurements from 6 females of *Photuris*. Slow flash data (from FF-I): 73 measurements from 6 males of *P. punctulatus*; 145 measurements from 10 females of *Photuris*.

Quick flash latency scarcely varies with temperature (Fig. 23). In the 10–30° range the Q_{10} for the slow flash varies between 1.9 and 2.2 whereas that for the quick flash ranges from 1.3 to 1.6. It is this fact which furnishes perhaps the clearest evidence of a basic mechanistic difference between the excitation processes of the slow and quick responses.

C. *Effects of repetitive stimulation.* Like the slow flash, the quick flash may augment with repetitive stimulation (Fig. 10) and may simulate the fusion phenomena of striated muscle (Figs. 11 and 12). As with the response of the slow flash to train simulation, there may be considerable differences in tetanizing frequencies between species (Figs. 12, 13) and even, apparently, between the sexes



FIGURES 24–46.

TABLE I

Approximate peripheral response latencies (msec.; 25°)

Species	Ultra-short	Quick flash	Slow flash*	Ultra-long
Woods Hole *Photuris*	1	18	70	
Maryland *Photinus pyralis*		20	160	
Photinus punctulatus		15	75, 110**	
Photinus consanguineus		10	100, 175†, 250†	
Photinus marginellus		5–10***	40–200	
Maryland and Iowa *Photuris* larva	1–3			600

* Values in FF-I, Table I, are for 22° as stated in text, not 25° as erroneously given in table heading.

** Differs with sex—See FF-I.

*** Slow flash values of 26 and 27 msec. given in FF-I, Table I, now thought to have been incompletely facilitated quick flashes.

† Secondary peaks, not appearing alone.

(Fig. 13 vs. 14). Paired-shock experiments indicate the same general course of facilitation as for the slow flash, with 10–15 msec. estimated as the intershock interval for maximum effect in *P. consanguineus* and less than 7 msec. in *P. punctulatus* (Fig. 22).

Repetitive evocation of quick flashes often induces a continuous homogeneous background glow just as does train stimulation of slow flashes (FF-I: Figs. 33–35, 37, 51, 62–66) and in fact most of the phenomena dealt with in the present paper were recorded against a background of continuous luminescence rather than of complete darkness. With both quick and slow flashes the detectability of individual

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FIGURES 24–46. (24) *Photinus consanguineus,* male, decapitated. First four frames are 1st, 3d, 5th and 7th consecutive responses to 5 msec./40 V. Last three frames are immediately following responses to 5 msec./50V., 5/60 and 5/70. S = 875. (25) As 16. Responses to 5 msec./15 V. and 30/150. S = 175. (26) Woods Hole *Photuris* larva, decapitated. Response to 30 msec. /150 V. S = 375. (27) As 26. Response to 10 msec./15 V. S = 2400. (28) Woods Hole *Photuris* larva, isolated organ. Response to train of 10 msec./100 V. shocks at 10/sec. S = 1750. (29) Iowa *Photuris* larva. Responses to (probably) 50 msec./150 V. S = 1080. (30) As 28. Three responses to 100 msec./150 V. S = 860. (31) Maryland *Photuris* larva, isolated organ. Response to train of 7 msec./150 V. shocks at 5/sec. Two sweeps (left end of upper trace continuous with right end of lower). S = 5500. (32) *Photinus consanguineus,* male, isolated 7th segmental organ. Response to 30 msec./150 V. S = 230. (33) As 32 except 6th segmental organ. (34) *Photinus consanguineus,* male, isolated abdomen. Response to 20 msec./150 V. S = 115. (35) *Photinus marginellus,* male intact. Response to 10 msec./150 V. S = 75. (36) *Photinus marginellus,* male isolated organ. Response to 10 msec./150 V. S = 75. (37) *Photinus consanguineus,* male, excised 6th segmental organ. Response to 10 msec./150 V. S = 88. (38) *Photinus marginellus,* male, decapitated. Response to 30 msec./150 V. S = 180. (39) *Photinus marginellus,* male. Response to 80 msec./150 V. S = 1750. (40) *Photinus punctulatus,* male, isolated 6th segmental organ. Response to 2.5 msec./100 V. S = 52. (41) Iowa *Photinus pyralis,* male, one half of one segmental organ freed of other viscera. Two successive responses to 10 msec./150 V. S = 47. (42) Iowa *Photinus pyralis,* male. Response to train of 10 msec./150 V. shocks at 12/sec. S = 925. (43) As 42. Response to 1 msec./150 V. S = 9.2. (44) As 43. Response to 0.1 msec./150 V. S = 4.5 (very fast sweep). (45) Moist filter paper. Three "responses" to 1 msec./150 V. S = 6. (46) Moist filter paper. "Responses" to 10 msec./150 V. and 100 msec./150 V. S = 47, 185.

responses to repetitive stimulation is limited by flash intensity because flash duration increases concomitantly and thus a flash tends to blanket or merge with preceding and succeeding peaks. The detection of frequency limits is also affected by the background glow that eventually develops. Assuming that the amount of glow is comparable in the two responses, and that flashes are approximately equal in intensity, the quick flash seems to have a markedly higher capacity for serial response than does the slow. The limit of 1:1 frequency response of the slow flash of the *Photuris* adult is 17–20/sec. (FF-I). The quick flash, in contrast, follows well at 40/sec. and apparently reaches its limit at something over 50/sec. Correspondingly, *Photinus pyralis,* which has real trouble in maintaining its slow flash at 4–5/sec., maintains its quick flash up to about 13/sec. For *P. punctulatus* the corresponding figures are 10 and more than 30/sec., and for *P. marginellus* they are 7 and 18/sec. These limits are roughly in the order of the respective species slow flash latencies (Table I). The contrast between the frequency compliances of slow flash and quick flash can be seen by comparing Figure 15 with Figure 13.

The quick flash is in general less stable than the slow and will not continue for hundreds or thousands of successive responses as may the adult slow flash. Nonetheless, under favorable conditions the fully facilitated quick flash may be quite reproducible (Fig. 16), making it possible to work out temperature effects and other data requiring repetitive stimulation. Fatigue or adaptation, when it does occur, may sometimes be overcome by intensifying the stimulus. For example, in tests on *P. marginellus* we found that flashing ceased after 20–30 successive responses to nominal 10 msec./150 V. stimuli given about a second apart, but would then resume at its original intensity for another 20–30 responses if the stimulus was changed to 20 msec./150 V., and so again at 30 msec./150 V.

Fatigue and adaptation also affect the interrelations of slow and quick flashes. Though the two flashes may occasionally be of equal magnitude (Figs. 1c, 7e) and may even facilitate together with repetitive stimulation (Fig. 17) it is more usual for one to come to predominate at the expense of the other (Figs. 7g, 7h). The quick flash seems to be favored by increasing either stimulus voltage (Fig. 5; Fig. 24e, f, g) or duration (Fig. 7). Changes may also occur with repetitive stimulation even without change in stimulus parameters. Usually it is the slow flash that drops out (Fig. 18), though if the stimulus is near the quick flash threshold the quick flash may disappear instead (Fig. 24a–d).

D. *Flash contour.* Quick and slow flashes may differ in form, but the fact that they may also resemble each other very closely, whether occurring separately (Fig. 25) or together (Fig. 7e), indicates that there is no *a priori* reason to suppose that the two effector events are basically different. A comparison of half-rise and half-fall times of quick and slow flashes of differing magnitudes likewise suggests that the two responses in a given individual are alike kinetically.

The quick flash, like the slow flash, may alter form somewhat under changing conditions of stimulation. There are some indications that the rise phase is slowed preferentially by low temperature and by increasing shock duration. The effects of increasing frequency have already been considered. Whether the continuous photogeny of the tetanized lantern or the background glow that is almost always present in quick flash experiments are comparable to any of the glow phenomena previously described (FF-I) is unknown.

3. *Flashing in relation to peripheral nerves*

Since nerves are inextricably and profusely incorporated in the lantern tissue there is no way, by stimulation alone, to test the possibility of peripheral nervous involvement in quick flash excitation. This problem was accordingly attacked by nerve section (see also Hanson, 1962).

In adult males of *Photuris* a transverse ventral cut was made at the boundary between abdominal segments 6 and 7 so as to leave segment 6 still innervated while destroying all connections between the nerve cord and the photogenic organ of segment 7. The animals were then carefully observed to make sure that spontaneous flashing still occurred in the segment 6 organ and not in the segment 7 organ. Then at intervals during the next 48 hours each segmental organ was tested separately for excitability, placing the electrodes directly in the photogenic tissue. In experiments with 15 specimens it was found that (a) the sixth segmental organ (control) of all individuals continued able to flash spontaneously and remained normally excitable for both slow and quick flashes throughout the experiment. (b) Up to about 20 hours after denervation, slow flashes with normal strength-duration relations, and also quick flashes, could be elicited from the segment 7 organ by appropriate stimuli (5 out of 5 specimens). (c) In the period 21–24 hours after operation, slow flash excitability was lost in 4 of 6 animals tested, quick flash excitability being retained in 5. (d) After 24 hours it was impossible, in the four remaining animals, to elicit a slow flash in the seventh segmental organ, though stronger stimulation did evoke the quick flash in each instance. As a control for the possibility that the loss of slow flash excitability in the denervated segment was due simply to inactivity of the lantern, a number of males were decapitated and tested for up to 8 days. They of course immediately ceased to flash spontaneously, but they remained able to produce both slow and quick flashes in both segmental light organs under appropriate electrical stimulation.

Since evidence from other insects indicates that it takes nerves a day or more to degenerate enough to lose their conductive powers, the present experiments could be interpreted to indicate that the normal slow flash is mediated by nerve, the quick flash not.

4. *Ultra-short latency responses*

A. *Larval flash.* In testing the isolated light organs of the *Photuris* larva to see if a short latency response similar to the adult quick flash could be induced, the effect of high voltage stimulation was found to be even more dramatic than in the adult (Fig. 26). The exact latency of the response is difficult to determine because of the low level of light emission but appears to be 1–3 msec. We shall refer to this response as the larval "flash" in distinction to the protracted normal larval "glow," which has a response latency of 600–800 msec. and a duration of 2–15 sec. (Fig. 27). Both flash and glow appear to involve the whole of the minute larval organ.

The larval flash facilitates with repetitive stimulation. The contrast in frequency compliance between short and long latency responses is even greater than in the adult, the normal glow being unable to follow stimuli one second apart (Chang, 1956; FF-I) whereas the flash shows clearly independent responses up to 10 stimuli per second (Fig. 28).

We have an occasional record indicating that the larval flash and larval glow may occur together—*i.e.,* records in which a temporary glow appears several hundred milliseconds after a flash. The strong stimulation commonly also induces a type of glow which begins at various brief intervals after the peak of the flash (Figs. 29, 30). Possibly this is analogous to the generalized glow that often follows intense stimulation of the adult lantern (FF-I, Figs. 63–66; FF-II, Fig. 43) and which may accompany intense serial stimulation of the larva (Fig. 31).

In some instances larval flash and glow seem to differ in contour, in that the flash shows a steeper rise than fall of luminescence (Fig. 26 vs. Fig. 27). In other instances (Fig. 30b) the flash resembles the normal glow in having an approximately symmetrical time course.

Larval flash latency seems to show the same relative independence of temperature as does that of the adult quick flash (Fig. 47).

In ten *Photuris* larvae the nerve leading to one of the pair of light organs was cut; then electrical excitability was tested in two larvae on each of days 1, 3, 5, 7 and 9 thereafter, with the normally innervated mate of each denervated organ serving as a control. Glows and flashes were both inducible on days 1 and 3, but after that only the flash was seen. It is concluded that the short latency response need not involve nerve.

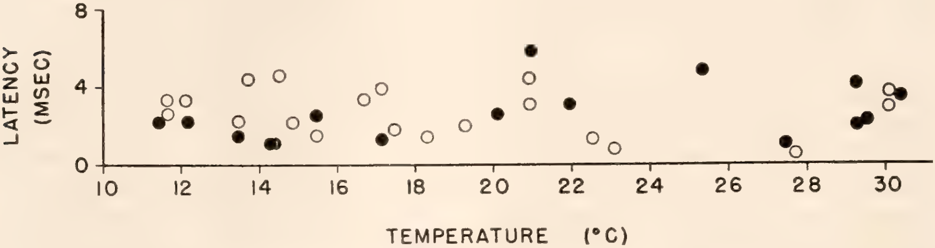


FIGURE 47. *Photuris* larval quick flash latency in relation to temperature. Thirty-six measurements on two individuals. Because of technical difficulties mentioned in text, the latency values are maxima.

B. *Adult quick-quick flash.* In experiments in which it was desired to exclude central nervous influence, lanterns were extirpated and laid across parallel electrodes in a moist chamber. With the small species, *Photinus consanguineus* and *P. marginellus,* the electrodes were often only about a millimeter apart because of small organ size. Under these conditions intense stimulation sometimes induced a dim and nearly instantaneous flash (Figs. 32, 34–38). This "quick-quick flash" was sometimes also obtained from large species of firefly if tissue fragments were used instead of whole lanterns (*P. punctulatus,* Fig. 40; *P. pyralis,* Fig. 41). The contrast between this response and the quick flash can be seen by comparing Figure 32 with Figure 33. Though sometimes occurring alone (Figs. 32, 38), the quick-quick flash usually merges with the quick flash either partially (Figs. 35, 36, 40) or completely (Figs. 34, 37, 41).

C. *Nature of ultra-short latency responses.* The fact that the flash of the larva and the quick-quick flash of the adult were only obtained with small pieces of tissue and high stimulus intensities suggests two possible explanations: Either the responses are due to a genuinely different type of pathway of excitation from those previously described, or they represent an artifactual heating or ignition of the tissue.

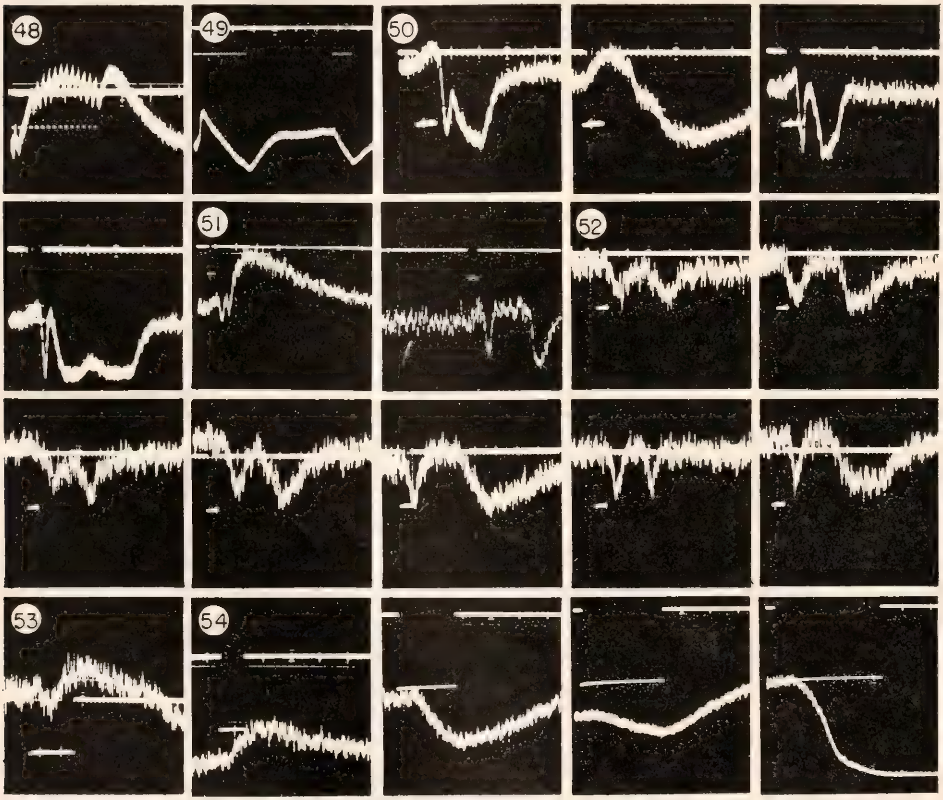
If the 1–3-msec. latency responses are biological rather than physical, the simplest explanation of the fact that they only appear under such special conditions would seem to be that the effect is one closely localized to the electrode, where the current flux is greatest. In other words, only when the total mass of responding tissue was small would the number of photocytes giving the very early response be large enough, in comparison with the number of cells giving slower types of response (quick or slow flash in adult; glow in larva), to become detectable. Detection would also be aided by the greater oscilloscope amplification necessitated by the small overall size of the tissue fragment. An analogous instance is the fact that a highly amplified record of a slow flash sometimes discloses a previously invisible leading shoulder (FF-I, Fig. 43). The fact that the ultra-short latency luminescences are always dim responses in the absolute sense is consistent with the idea that they reflect the activity of relatively few photocytes. Further, a biological nature for the quick-quick flash seems favored by its usual variability in relation to the quick flash (see above) and in relation to duration of stimulus pulse (Figs. 32, 34–40).

Considering now the artifact possibility, the fact that the quick-quick flash of some preparations can maintain its form practically unchanged through a dozen or more successive stimulations seems incompatible with the idea that a progressive charring of photogenic tissues is taking place. On a single cell basis the intensity of stimulation may be by no means as physiologically outrageous as might appear from the nominal stimulus parameters. However, in order to have an unequivocal artifact for comparison we "stimulated" small pieces of filter paper wet with saline. The response obtained, which we shall call a "spark," was of apparently zero latency but was quite different in some respects from most quick-quick flashes. For example, sparks were obtainable with stimulus pulses of 1 msec. or even shorter duration, which is never true for the quick-quick flash or larval flash. Also, the rise and decay phases of the luminescence were much faster than those of the responses obtained from photogenic tissue, so that duration was scarcely longer than the stimulus pulse (Fig. 45). With 10 msec. or longer stimuli sputtering occurred (Fig. 46) rather than the relatively long-lasting luminescence usual with tissue (Fig. 32, 34–41). However, in one lantern fragment preparation that had been used repeatedly, effects similar to sparks were obtained (Figs. 42–44).

If ignition of filter paper does occur, as suggested by Figures 45 and 46, the same effect probably would sometimes also occur in tissue. It seems possible that because of their extremely fast rise, 1–2-msec. duration and presumed limitation to a zone very close to the cathode, sparks would escape detection in records made at the oscilloscope sweep speeds ordinarily used and with appreciable masses of tissue. This might explain the absence of any spark-like element in Figures 32 and 34–41, while still allowing for occurrence of tissue sparks under extreme conditions (Figs. 42–44). However, it must be admitted that the biological significance of the quick-quick flash in the adult, and perhaps also of the larval flash, is questionable.

5. *Quenching phenomena*

In our first paper we showed that the continuous glowing that often develops in the lantern during and after vigorous repetitive induction of the slow flash can show either enhancement or depression both during and after electrical stimulation. The glows that arise during the evocation of quick and quick-quick flashes (see section 2C above) may likewise vary in intensity both spontaneously and as a consequence of stimulation, showing relatively slow enhancement and reduction (Figs. 48, 49) very reminiscent of the responses produced by weaker shocks in our previous investigation (*e.g.*, FF-I: Figs. 60, 65). We have also observed a curious phenomenon in the larva, in that baseline luminescence after the flash sometimes sinks to a lower level than preceding it (Figs. 26; 30c).



FIGURES 48–54. (48) *Photinus marginellus*, male, decapitated. Response to train of 10 msec./150 V. shocks at 10/sec. S = 3200. (49) As 48, but train frequency 15/sec. (two bursts). S = 4300. *Photinus marginellus*, female, decapitated. Four successive responses to 30 msec./150 V. S = 325. (51) *Photinus marginellus*, female, decapitated. Two responses to shocks of 10 msec./150 V. S = 425. (52) *Photinus marginellus*, male, decapitated. First, 5th, 6th, 9th, 13th, 14th and 15th successive responses to shocks of 10 msec./150 V. at 1.5 sec. intervals, after fatiguing. S = 175. (53) As 52 except 40 msec./150 V. S = 175. (54) *Photinus marginellus*, female, decapitated. Responses to 15 msec./150 V., 100/150 and 400/150. S = 130, 312, 625, 625.

From lanterns that have been so thoroughly fatigued by intense repetitive stimulation that they no longer flash, but show a steady dull glow, we have recorded some remarkable quenching responses that are comparable kinetically with flashes. Records from three individuals are shown in Figures 50–53. The response often begins with a slight increase in luminescence of near zero latency, presumably a quick-quick flash. After 5–20 msec. this is abruptly interrupted by the first quenching episode, which requires 10–15 msec. to reach maximum dimming (which is not complete extinction) and terminates in 20–25 msec. Almost immediately thereafter the light dims for a second time. Actually, it is unclear whether the overall phenomenon represents two brief quenchings or one prolonged quenching with a superimposed flash, but in any case the most interesting feature of the response is the initial short-latency dousing. The influence of stimulus duration on quenching is shown in Figure 54. Luminescence terminates in irreversible and complete "burnout" when the stimulus reaches 400 msec./150 volts (Fig. 54d). Like most of the other high voltage effects, the quenching changes are small in absolute intensity, suggesting a phenomenon closely localized to the electrode position.

DISCUSSION

In our second paper we described a number of classes of long-latency response in the adult firefly, involving possible multiple cord conduction rates, central junctional delays, and central reverberation. In the present paper we have described two additional types of response, the quick and the quick-quick flashes, both with conspicuously shorter latencies than that of the normal slow flash. These responses need to be identified and integrated into a unified scheme of excitation. In attempting this synthesis from our exploration of a system hitherto essentially unknown electrophysiologically, we have had to adopt a somewhat more speculative approach than if the firefly lantern preparation had reached the conventional single unit nerve-junction-effector stage.

A. *Lantern microstructure.* Until quite recently it was not possible to advance much in the way of a concrete and coherent model of the excitation route in the firefly because the termination of peripheral nerve in the lantern was unknown and even the site of light production was in dispute. It is still not absolutely certain that the light is produced in, or at least only in, the so-called photocytes, although this is the traditional view and the one we shall adopt for present purposes. However, the innervation problem has been settled recently by the exquisite electron micrographs of Smith (1963) showing that the ultimate fiber, about 0.3 μ in diameter, does not touch photocytes at any point. Rather it terminates in a reticule-like ending containing both synaptic vesicles and neurosecretory-like droplets which embraces the mitochondria-filled tracheolar cell and is in turn embraced by the tracheal end-cell. This intricate structure, strategically situated just proximal to and in contact with the photocyte, near the transition point between terminal tracheal twig and ultimate tracheoles, has long been suspected of being involved in flash control though not as a neuroeffector link (see reviews in Buck, 1948; Hastings and Buck, 1956). For the purpose of our present speculations it will be assumed that this "end-organ" functions as a neuroeffector junction in the transfer of excitation in a three-element linkage: peripheral nerve-end organ-photocyte.

B. *Suggested nature of short-latency responses.* Considering, first, the identity of the adult quick flash, two main interpretations seem possible. Either it represents the response of a different group of photocytes from those producing the slow flash or it represents a response of the same cells triggered by a different process or pathway. We have not been able to exclude the former alternative but feel that essentially all our data favor the view that slow and quick flash represent the same effector event. These data include the close similarity of the two flashes in contour (Figs. 7e, 25), the apparent origin from the same tissue, and the mutual capacities for showing facilitation, summation, fatigue and other responses typical of a neuroeffector. The quantitative differences in threshold and in latency can be attributed to excitation differences.

The other short latency response of the adult firefly, the quick-quick flash, has much less secure physiological status than the quick flash, and we have far fewer data concerning it. Its usual kinetic difference from the induced filter paper spark, its variability, and its repetitivity, argue against its being a pure artifact. We suggest, primarily on the basis of its short latency and relative dimness, that it represents direct stimulation of a relatively small number of photocytes. We suggest the same explanation for the ultra-short latency larval flash. The fact that the larva lacks an intermediate latency response, corresponding to the adult quick flash, might seem an obstacle to the proposed scheme. Rather, it is a point in support, because the larval light organ differs from that of the adult in lacking tracheal end-organs.

We thus tentatively propose that slow, quick and quick-quick flashes all represent basically the same effector event; and that the overall response latency therefore comprises a delay representing peripheral nervous conduction, a delay involving nerve ending-end organ interaction, a delay due to activities of the tracheal end organ, and a delay during the ultimate excitation of the photocyte.

C. *Hypothetical origin of latency classes.* If, in the proposed chain of excitation, the thresholds to external stimulation of the three individual links are in the order photocyte > end organ > peripheral nerve, only nerve will be excited by a minimal stimulus. The latency of the eventual flash, involving conduction delay of nerve, junctional delays and photocyte activation time, will then be maximal (slow flash). If the stimulus strength is sufficiently increased, which can be accomplished either by increasing the voltage and/or stimulus duration or by reducing tissue mass, the thresholds of both end-organ and nerve to direct excitation will be exceeded. Thereupon two responses may occur. One involves the complete excitation chain (slow flash), and the other, skipping now the neural link, involves just the end organ-to-photocyte portion (quick flash). Finally, a still stronger external stimulus could by-pass both nerve and end organ, stimulating the photocyte directly (quick-quick flash). Depending on the interplay of stimulus strength, degrees of facilitation and fatigue, and tissue disposition in relation to electrode, the three responses might theoretically occur alone or in combination. Quick and slow flashes are usually easily differentiated instrumentally and in some instances (*e.g., P. pyralis*) even by eye. The quick-quick response, however, is not visually distinguishable from the quick flash, since the two latencies are so close, the intensity of the quick-quick flash so small, and the diffusion of light through adjacent non-excited photogenic tissue so prevalent. The quick-quick flash would, in fact, not be detectable even in the oscilloscope record unless the light signal was strongly amplified or the tissue mass was sufficiently small that the directly excited photocytes in the region of most intense current flux close to the electrode formed an appreciable fraction of those excited through additional links.

D. *Evidence concerning by-pass hypothesis.* In evaluating the hypothesis that the three progressively shorter latencies are due to by-passing successive links in the excitation chain by increasingly intense stimulation, we shall use the approximate latency measurements from *Photuris* at 25°, namely 1 msec. for adult quick-quick flash and larval flash, 18 msec. for the quick flash, and 70 msec. for the slow flash.

The provisional identification of the quick-quick flash with direct excitation of the photocyte is supported only by the fact that its latency is of the right order for a process such as momentary depolarization of a membrane.

A distinction between quick and slow flashes is strongly supported by the nerve section experiments and the temperature data. The former indicate that the slow flash depends on nerve, the quick flash not. The latter shows that below 30° slow flash excitation changes exponentially with a Q_{10} of 2 or higher, whereas the quick flash Q_{10} averages around 1.4. If quick flash excitation were independent of conduction delay it would also be understandable why its frequency compliance can be so much higher than that of the slow flash. The 8-msec. quick flash latency is certainly inordinately long for junctional or end plate delays in known neuro-effector systems, although it might be reasonable for a neuro-glandular effect. Smith's study shows that the firefly end-organ is unique in that the nerve terminals are separated from the presumed ultimate effector, the photocyte, by another cell type, the peritracheal cell. However it seems unlikely that diffusion across even this gap of 1 μ or more (plus three cell membranes) could account for the otherwise excessive delay.

Turning finally, to the approximately 50-msec. differences between slow and quick flash latencies, this delay might represent either conduction alone or conduction plus some part of end-organ activation. The former is the simpler choice and it is by no means excluded, but we have already shown (Introduction) that there are considerable obstacles to assigning more than a few milliseconds of the total latency to pure conduction. In particular it would certainly be expected that some of the cells in a lantern responding to direct stimulation would be too close to the electrode to require anything like a minimum of 50 msec. for conduction of the stimulus to them. It seems likely, therefore, that the delay involved in overall end-organ and junction activation includes not only the 18 msec. suggested above but a considerable fraction of the slow flash latency. This implied complexity of excitation is consistent with the very complex end-organ morphology disclosed by Smith's study.

E. *Extinction mechanisms.* Historically, most of the attention to the firefly flash has been focused on the initiation and rise of luminescence, it having been assumed tacitly that the decay phase, like that of the *in vitro* "flash," merely reflects the chemical decay of an aliquot of reactants brought together by the excitation, or at most some sort of product or feedback inhibition. Buck (1948, 1955) did indeed speculate on the possibility that the firefly expends energy in keeping itself dark, flashing then representing a transient release from this inhibition, but this idea was based on the uncontrolled glow that develops in hypoxia, anesthesia, and death, rather than on any specific neural mechanism. Although rise and fall of luminescence seem to be affected differently by temperature and hypoxia (FF-I, II), there are few indications that the falling phase of the flash is due to a specific extinguishing mechanism.

The rapid quenching phenomenon discovered in the present investigation, involving intense stimulation, fatigued tissue, and low-intensity light (hence probably an effect closely confined to the electrode region), is not likely to be related directly to the decay phase of the normal flash. As a reversible extinction mechanism it is nevertheless of great theoretical interest. Our previous demonstration of central inhibition of flashing by eye stimulation (FF-II) is irrelevant in the present connection, since it refers to a process that prevents the flash from starting, rather than one that snuffs out a glow already in being, but there would seem to be no *a priori* prohibition to some sort of peripheral inhibition, for example, one with a higher threshold and greater resistance to fatigue than the excitatory process.

The complex excitation path suggested by our findings indicates that study of single units will be necessary before much further progress can be expected. Preliminary studies have shown that the lantern tissue is held together so tenaciously by its profuse network of tracheoles that all usual dissociation methods fail. Hence it will probably be difficult to isolate single neuron-end organ-photocyte units. However, it has been found possible by localized stimulation to excite one or a few photocytes individually, and though these cells are inconveniently small (10 μ in largest dimension) it should be possible to record intracellularly.

SUMMARY

1. Recordings of flashes from minute, widely separated regions of the lantern indicate that peripheral excitation is rather uniform.

2. Evidence is presented that peripheral conduction is unlikely to account for more than a small proportion of overall response latency.

3. By stimulating lantern tissue directly with high intensity shocks it is possible to evoke a much earlier response instead of or in addition to the normal flash. For example, in *Photuris* this "quick flash" occurs about 18 msec. after stimulation as compared with about 70 msec. for the normal or slow flash (at 25°).

4. The quick flash resembles the slow in contour, in apparently being produced by the same tissue, in showing a strength-duration effect for threshold and in showing facilitation, summation, adaptation, fatigue and other typical neuroeffector responses. The quick flash differs from the slow in its shorter latency, higher threshold, higher frequency compliance, and in having a relatively temperature-insensitive response latency.

5. Nerve section experiments show that the slow flash depends on nerves, the quick flash does not.

6. In the small larval light organ and in small pieces of adult lantern, very intense stimulation may induce a flash with a latency of the order of 1 msec. Control experiments with wet filter paper indicate that this "quick quick" flash is not entirely an artifact.

7. It is hypothetically proposed that stimulation involves a three-element neuroeffector (nerve terminal, end organ, photocyte) and that increasing strengths of

stimulus can by-pass in stepwise fashion certain links in this chain of peripheral excitation. According to this idea the ultrashort ("quick quick") response would represent direct excitation of the photocyte, the quick flash latency would represent delay necessary for excitation of the photocyte by the tracheal end organ, and the difference between slow flash and quick flash latencies would be time occupied in activation of the end organ complex plus a small delay for peripheral nervous conduction.

8. In lanterns subjected to long-continued intense stimulation the flash eventually disappears and is replaced by a steady glow. Under some circumstances intense shocks produce striking momentary double quenchings of this glow, kinetically similar to a flash. Peripheral inhibition is suggested as a possible mechanism.

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THE MODIFICATION OF REPRODUCTION IN INSECTS TREATED WITH ALKYLATING AGENTS. I. INHIBITION OF OVARIAN GROWTH AND EGG PRODUCTION AND HATCHABILITY

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Chemical compounds that can induce sexual sterility in the screw-worm fly (*Cochliomyia hominivorax* (Coquerel); Diptera, Calliphoridae) have received intensive study during the past two years. Such compounds have been termed "chemosterilants," regardless of their mode of inducing sterility. Chemical sterilization of screw-worm flies is of interest in a program of control or eradication of this species by the use of sexually sterilized males. Screw-worm flies have been successfully eradicated from Curaçao and the southeastern United States by the release of males rendered sexually sterile by a physical agent, ionizing radiation (Baumhover *et al.,* 1955; Knipling, 1960). The development of an effective chemical substitute for radiation might offer the additional advantages of overcoming the adverse biological effects of radiation sterilization and/or reducing the considerable mechanical handling of the flies.

Among chemical agents, biological alkylating agents appear to offer the greatest promise of success as chemosterilants against screw-worn flies (Crystal, 1963). A biological alkylating agent is a compound that can effect the addition of an alkyl group or a compound radical, with or without the replacement of a hydrogen atom, in biologically significant functional groups under physiological conditions. Various aliphatic, aromatic, and heterocyclic analogues of ethylenimine, all aziridinyl compounds, are biological alkylating agents that possess outstanding chemosterilant activity. In an earlier report (LaChance and Bruns, 1963), data were presented on the measurements of ovaries from females subjected to gamma radiation. It was found that the growth of ovaries of newly emerged females subjected to gamma radiation was much more likely to be inhibited than that in 24-hour-old or older females similarly treated. This pattern of radiation-induced sterility in female screw-worm flies forms the basis for the present comparisons of the effects of chemical sterilization. A report of the effects of selected chemosterilants on the female screw-worm fly with respect to ovarian growth and egg production and hatchability follows.

Materials and Methods

Laboratory-reared screw-worm flies are ready to mate when they are two days old, and three or four days after mating each female lays about 200–250 eggs in a shingle-like mass. The newly emerged female fly possesses a pair of immature ovaries, each consisting of 100–150 ovarioles. Maturation in all ovarioles occurs synchronously, and each ovariole produces a mature egg in the first egg mass.

In these tests each series of determinations was conducted with flies from a single rearing that emerged from 8 AM to 12 noon on the first morning of the test.

The flies were sexed and the males set aside for later use. Females less than four hours old were treated with chemosterilants the morning that they emerged; females 24 ± 2 hours old were treated the next morning. A group of females was left untreated and used as controls. The treated and control flies were caged in a colony room maintained at 80° F. and fed water and undiluted honey.

Five aziridinyl chemosterilants were used in the treatments of flies. These were: One bifunctional compound, 2,5-bis(1-aziridinyl)-3,6-bis(2-methoxyethoxy)-*p*-benzoquinone; three trifunctional compounds, thiotepa (tris(1-aziridinyl)phosphine sulfide), tretamine (2,4,6-tris(1-aziridinyl)-*s*-triazine), and methyl tretamine (2,4,6-tris(2-methyl-1-aziridinyl)-*s*-triazine); and one hexafunctional compound, apholate (2,2,4,4,6,6-hexahydro-2,2,4,4,6,6-hexakis(1-aziridinyl)-1,3,5,2,4,6-triazatriphosphorine). The dosage of each compound, shown in Tables I and II, was that which had previously elicited complete or nearly complete sterility in females treated when 0–24 hours old and mated with untreated males (Chamberlain, 1962; Crystal, 1963).

TABLE I

Growth of ovaries in 6-day-old screw-worm flies treated with chemosterilants at 0–4 or 24 hours after emergence

Chemosterilant*	Series	Mean volume (mm.^{3}) of right ovaries with standard error from**		
		Females treated at age		Untreated
		0–4 hours	24 ± 2 hours	
Benzoquinone derivative	1	0.735 ± 0.090 a***	4.15 ± 0.500 b	9.39 ± 0.435 c
	2	1.37 ± 0.192 a	4.58 ± 0.466 b	8.18 ± 0.115 c
Thiotepa	1	4.72 ± 0.640 a	6.63 ± 0.517 b	8.51 ± 0.374 c
	2	—	6.39 ± 0.386 a	7.31 ± 0.344 a
	3	6.97 ± 0.482 a	8.17 ± 0.372 a	6.29 ± 0.429 b
Tretamine	1	2.41 ± 0.288 a	7.80 ± 0.361 b	9.12 ± 0.330 c
	2	1.56 ± 0.296 a	7.01 ± 0.492 b	8.03 ± 0.256 b
Methyl tretamine	1	—	5.26 ± 0.564 a	8.28 ± 0.397 b
	2	0.91 ± 0.039 a	3.44 ± 0.514 b	6.29 ± 0.429 c

* Each fly treated topically on the dorsal thorax with 2 μl. of 0.5% for the first three compounds and 2% for methyl tretamine; tretamine in methanol, others in acetone.

** Twenty to thirty pairs of ovaries measured per treatment except 12 pairs in thiotepa series 1, 0–4 hours old.

*** Means within a row followed by the same letter are not significantly different from each other at the 5% level.

Female flies were treated by the topical application of solutions of chemosterilants to the dorsal thorax with a micrometer-controlled calibrated syringe. Two microliters were applied to each fly, anesthetized by chilling, except in the first tretamine-treated group to which 2.2 μl. were delivered. In the earlier experiments, the mechanical manipulation and anesthesia of the flies, especially of the younger

TABLE II

Female fertility, fecundity, and egg hatchability data from tests with screw-worm flies treated with chemosterilants at 0–4 or 24 hours after emergence

Chemosterilant*	Series	Fertility** $\left(\frac{\text{♀♀ ovipositing}}{\text{♀♀ egged}} \times 100\right)$	Fecundity*** $\left(\frac{\text{Eggs/treated ♀}}{\text{Eggs/control ♀}} \times 100\right)$	Egg hatchability*** (%)
Treatment at 0–4 hours of age				
Benzoquinone derivative	2	0	—	—
Thiotepa	2	5	96	43
	3	53	72	40
Tretamine	2	2	73	100
Methyl tretamine	2	0	—	—
Apholate	1	70	89	85
	2	65	100	49
Treatment at 24 ± 2 hours of age				
Benzoquinone derivative	1	20	82	31
	2	35	89	3
Thiotepa	2	62	100	82
	3	71	100	79
Tretamine	2	60	100	0
Methyl tretamine	1	22	89	5
	2	21	68	0
Apholate	1	88	86	100
	2	92	100	90

* Each fly treated topically on the dorsal thorax with 2 μl. of 0.5% for the first three compounds and 2% for methyl tretamine in acetone except tretamine in methanol. Apholate: series 1, 10% in 1% aqueous Tween 20; series 2, 5% in methanol.

** Twenty-two to sixty-five females per treatment given the opportunity to oviposit.

*** Mated with untreated males at 5 days of age. Corrected for untreated controls taken as 100%; 1,500–3,000 eggs from fertilized females scored per series, except in tretamine treatment of 0- to 4-hour-old flies in which one female laid one mass of 152 eggs.

groups, resulted in severe adverse effects which were reflected in extremely poor survival. In some instances, there were enough survivors only for ovarian measurements or egg-production determinations, but not both. Therefore, in most of the later tests, great efforts were made to handle the flies very gently and to submit them to the minimum time of exposure to cold necessary for sexing treatment.

When the flies were 5 days old, males were added to the females' cages at 4 PM. The next morning, 17 hours later, the females were given the opportunity to lay eggs and the rate of oviposition and percentage of hatch were determined as previously described (LaChance and Leverich, 1962). At 6 days of age, some females from each treated and control group were killed and preserved in 70% ethyl alcohol for measurement of the ovaries. As described elsewhere (LaChance and Bruns, 1963), the product of the maximum length, width, and depth of each ovary gave an approximate volume which was sufficiently accurate for comparisons between groups.

RESULTS

Ovarian growth

The data on ovarian measurements are given in Table I; each row represents a series of measurements of ovaries from 6-day-old females treated at the two different ages. Only determinations of right ovaries are included, since data for left ovaries were essentially identical. Differences between the mean ovarian volume of 0- to 4-hour-old flies and 24-hour-old flies, and of 24-hour-old flies and control flies, were tested for significance by the Student's *"t"* test. Those having a *P*-value of 0.05 or less have been so labeled in the table.

Reference to Table II reveals that all compounds but apholate were highly effective in their ability to inhibit egg production in very young flies. Because apholate was largely without effect as an antifertility agent in young female screw-worm flies, it was not expected that ovarian growth would be greatly inhibited. Measurements of the ovaries of females treated with this compound were, therefore, not made.

In both series of determinations with 2,5-bis(1-aziridinyl)-3,6-bis(2-methoxyethoxy)-*p*-benzoquinone, the responses of screw-worm fly ovaries were very similar (Table I). Little ovarian growth was evident when newly emerged flies were treated. Significant retardation—about 50%—of the growth of ovaries was effected by the compound when flies were treated when 24 hours old. The ovaries of flies treated at 0–4 hours after emergence were all small—50–75% of them were less than 1 mm.^{3}. Those of flies treated at 24 hours of age were distributed over a wide range of sizes; some were small, some intermediate, and some large. The measurements of ovaries of untreated flies were generally large—95% were greater than 7 mm.^{3}.

Thiotepa, tretamine, and methyl tretamine were not as uniformly effective as the benzoquinone compound. Of three replicates with thiotepa (Table I), only the first series showed a trend similar to that produced by the benzoquinone derivative. In series 1, the ovaries of newly emerged flies treated with thiotepa were about equal in size to those of 24-hour-old flies treated with the benzoquinone chemical and about half the size of those of untreated flies. The measurements of these ovaries varied widely, somewhat like those of 24-hour-old flies treated with the benzoquinone compound. Ovaries of 24-hour-old flies receiving thiotepa were intermediate in size between those of the youngest treated group and the untreated group. Measurements of ovaries of flies treated at 24 hours of age were also distributed over a wide range, but with a greater incidence of larger measurements. Nevertheless, the mean ovarian volumes of the three groups were significantly different from each other. In series 2, survival of all flies, including males set aside for later mating, was very poor. In part, this poor survival was due to overchilling and excessive handling. It is also possible that the flies of this rearing were of low vigor, as occurs from time to time in the laboratory rearing of screw-worm flies. No young flies survived for ovarian measurements, and flies treated at 24 hours of age and control flies had ovaries very nearly alike in size. Similarly, the third series of treatments produced effects not greatly different from each other. It is thought that the small mean volume of control ovaries in this series may have been due to overcrowding in the emergence cages. Flies for treatments were removed from emergence cages as needed. The number of pupae placed in such cages varied from test to test, and the number of flies emerging may have also varied. It is suggested that the number of flies present in the emergence cage and from which the control flies were obtained was too great for the volume of the cage. Competitive stresses, greater than in noncrowded conditions, were set up which retarded the development of the control flies.

Tretamine exerted inhibiting effects on ovarian growth that were different from those produced by the first two compounds (Table I). There was a greater range of ovarian measurements with this compound than with the benzoquinone compound, but not as large as with thiotepa. At the time of the first series of tretamine tests, which was the first series chronologically, flies 48 hours old were also included. However, the mean volume of right ovaries was 8.02 ± 0.327 mm.^{3}, not significantly different from those of flies treated at 24 hours of age or of untreated flies. It was concluded that ovarian response of 24- and 48-hour-old flies in series 1 was similar, and further tests with this age group were omitted. The second series of tretamine treatments appeared to produce effects very similar to the first with respect to newly emerged and 24-hour-old flies. However, the control ovaries did not develop to the extent of those in the first replicate, perhaps due to a reduction in fly vigor or to fluctuation in environmental temperature or to overcrowding in the cages. Consequently, the means of ovarian volumes of flies treated at 24 hours and of untreated flies were not significantly different from each other.

The data of the first treatment series with methyl tretamine (2%) resemble those with the first thiotepa treatment series (0.5%) (Table I). The data of the second treatment series with methyl tretamine more closely resemble those produced by the benzoquinone derivative. Only two flies treated at 0–4 hours of age survived the first treatment; the mean ovarian volume was 4 mm.^{3}. The same general distribution of ovarian measurements was present as with the benzoquinone compound.

Egg production and hatchability

Five alkylating agents were tested for their ability to inhibit egg production in screw-worm flies treated when 0–4 hours old or 24 hours old. In some treatments, high rates of mortality, perhaps due to cold sensitivity during anesthesia, especially among newly emerged flies, resulted in reduced numbers of survivors. It is seen in Table II that the first series of treatments with the benzoquinone compound and with methyl tretamine of 0- to 4-hour-old flies are lacking. The series numbers in this table correspond to those of Table I; series numbered alike in the two tables were treated at the same time. All of the surviving flies were used for the determinations of ovarian growth and no flies were egged in the benzoquinone and methyl tretamine series absent in Table II. The authors believe that the survivors recovered adequately from the effects of anesthesia and that the measurements of the ovaries of these flies are valid. Thiotepa-treated flies in series 1 (Table I) were subjected to stress as a result of the failure of relative humidity control, and egg production determinations of tretamine-treated flies in series 1 (Table I) were not planned. Therefore, among data for flies 0–4 hours old in Table II, there is no series 1 to relate to the correspondingly numbered series in Table I.

Females treated within four hours following emergence with the benzoquinone compound and with methyl tretamine failed to lay any eggs (Table II). Only 2% (one of 50 flies) treated with tretamine produced eggs. In one trial with thiotepa (series 2), almost all females within this age group failed to lay eggs whereas about half of those in series 3 did so. A 10% aqueous solution of apholate, which wetted the flies poorly, had the same effect as a 5% solution in methanol. Even though wetting was greatly improved with methanol, two-thirds of the flies treated at 0–4 hours of age laid eggs after treatment with either solution. Although very few flies laid eggs after some treatments with chemosterilants, those that did produced about 70% or more of normal numbers.

When screw-worm flies were treated at 24 hours of age, eggs were laid by at least 20% of the flies regardless of the chemical used. The benzoquinone compound and methyl tretamine exerted the greatest inhibitory effects on oviposition. With regard to oviposition both thiotepa and tretamine were two to three times less effective than the benzoquinone compound or methyl tretamine, and apholate was essentially without effect. In most instances, the egg production of flies was substantially normal.

Another area of influence of alkylating agents, their ability to induce dominant lethal mutations, was reflected in the hatchability of the eggs laid, as shown in the last column of Table II. When newly emerged flies were treated with the benzoquinone compound (0.5%) and methyl tretamine (2%), oviposition was completely inhibited. However, eggs laid by flies treated with these compounds at one day of age were largely nonviable, which indicated a high degree of efficiency of these compounds in inducing dominant lethal mutations in 24-hour-old flies. Thiotepa and apholate were moderately effective with newly emerged flies but largely ineffective with the older group. Tretamine was ineffective with young flies but completely effective with the older flies. Thus, dominant lethal mutations among 24-hour-old flies were extensive following treatment with the benzoquinone compound, tretamine, or methyl tretamine. The activity of biological alkylating agents as mutagenic agents in older flies is reported and discussed in greater detail in a companion report (LaChance and Crystal, 1963).

Discussion and Conclusions

The data presented above show that the effects of aziridinyl compounds on the reproductive potential of female screw-worm flies were not unlike those of gamma radiation. LaChance and Bruns (1963) demonstrated that the stage of development of the ovarioles at the time of irradiation determined the extent to which ovarian growth was affected. The most radiosensitive stage occurred when the egg chambers contained nurse cells undergoing endomitotic replications of chromosomal material. In the female this stage corresponded to the period of adult life within four hours of emergence. When delivered during this interval of development, the same dose of radiation was much more likely to cause infecundity than when delivered to 24-hour-old adults after endomitosis was completed. With the four chemosterilants tested, the greatest inhibition of ovarian growth also occurred during the endomitotic phase of the nurse cells (0–4 hours) and resulted in complete or nearly complete infecundity. The growth of ovaries of 24-hour-old females was but slightly or moderately affected, and the fecundity of such females was correspondingly greater than that of newly emerged females. Thus, at least with respect to the effects of these compounds on ovarian growth and fecundity of female screw-worm flies, the term "radiomimetic" is quite appropriately applied.

In no instance did treatment with a chemosterilant effect a complete cessation of ovarian growth. The ovaries of normal 24-hour-old females were previously found to attain a mean volume of about 0.2 mm.^{3} (LaChance and Bruns, 1963). The smallest ovaries found among chemically treated flies were more than four times this size by the time the flies were 6 days of age. It is tempting to seek to establish a relationship between the effects of radiation (LaChance and Bruns, 1963) and those of radiomimetic chemicals. The benzoquinone compound, at a dose of 10 micrograms, produced the greatest inhibition of ovarian growth at either age of treatment. The mean volumes of ovaries from flies treated at 0–4 hours and at 24 hours after emergence were about 1 and 4 mm.^{3}, respectively (Table I). The mean volume of ovaries from 0- to 4-hour-old flies exposed to gamma radiation approached 1 mm.^{3} as the radiation dose was increased beyond 4,000 r (LaChance and Bruns, 1963). LaChance and Bruns (1963) did not report that 24-hour-old flies were irradiated, but they did expose 48-hour-old flies to various doses. It was mentioned earlier in the present report that the inhibition of ovarian growth by tretamine treatment of 24-hour-old and 48-hour-old flies was very similar. The authors believe that the response of 24-hour-old and 48-hour-old flies to gamma radiation would also be similar to each other. The mean volume of ovaries of 48-hour-old flies was not reduced below 6 mm.^{3} when flies of this age were exposed to gamma radiation between 2,000 and 8,000 r. Therefore, the treatment of 0- to 4-hour-old flies with 10 micrograms of the benzoquinone derivative or with at least 4,000 r gave about the same degree of ovarian inhibition. However, the treatment of 24-hour-old flies with 10 micrograms of the benzoquinone compound resulted in ovaries about 4 mm.^{3} in volume whereas the treatment of 48-hour-old flies with 8,000 r resulted in a mean ovarian volume of about 7 mm.^{3}. It is suggested from these results that, at these dosage levels, 24- to 48-hour-old flies were almost twice as resistant to radiation inhibition of ovarian growth as they were to chemical inhibition. Although this is a speculative conclusion based on relatively few data, it would be of interest to examine the relationship further to determine whether it is a real one. Among newly emerged flies, thiotepa was the least active chemosterilant—the mean ovarian volume in series 1 and 3 was approximately 5–7 mm.^{3} (Table I). Tretamine was the least active chemosterilant when 24-hour-old flies were treated and the mean ovarian volume of these flies was about 7 mm.^{3}. The exposure of flies 0–4 hours and 48 hours hours of age to 2,000 r resulted in ovaries of about 4 and 7 mm.^{3}, respectively (LaChance and Bruns, 1963). Therefore, the inhibition of ovarian growth induced by thiotepa in 0- to 4-hour-old flies and by tretamine in 24-hour-old flies produced effects corresponding to those elicited by 2,000 r or less.

The following tabulation shows the relative antifertility effects previously reported (Crystal, 1963; Chamberlain, 1962) for the five aziridinyl compounds utilized in the present study. Female screw-worm flies, less than 24 hours old, were treated topically with the indicated doses of chemosterilant and mated with untreated males. The percentages of fecundity and egg hatchability have been corrected for the control percentages, taken as 100%.

Chemosterilant	Dose (μg.)	Fecundity $\left(\frac{\text{Eggs/treated } ♀}{\text{Eggs/control } ♀} \times 100\right)$	Egg hatchability (%)
Benzoquinone derivative	10	41	6
Thiotepa	12	0	—
Tretamine	11	9	0
Methyl tretamine	46	0	—
Apholate	300	75	1

At equivalent doses, the bifunctional benzoquinone derivative was incompletely effective and the trifunctional thiotepa and tretamine were equally effective as chemosterilants. Four times as much methyl tretamine as any one of these three compounds was required to produce complete sterility, whereas 30 times as much apholate was not quite completely effective. The relative inefficiency in these tests of the benzoquinone compound in preventing egg production or hatch stands in contrast to its effectiveness as an inhibitor of ovarian growth. The data reveal that the nature of the response to treatment with this chemical was dependent on the age of the fly. Only flies treated at 0–4 hours failed to lay any eggs (Table II). When older flies were treated, they laid 41% as many eggs as control flies (Crystal, 1963). Treatment at 24 hours of age resulted in 82% as many eggs being laid by treated flies as by control flies, and the ovaries of the treated flies were half as large as those of controls (Tables I and II). Tretamine is another example of a compound that was an effective inhibitor of ovarian growth in young females (Table I) but had little effect on older females, as seen in the high egg production of treated 24-hour-old females (Table II). This compound, in contrast to the benzoquinone compound, induced 100% dominant lethal mutations and was, therefore, an effective chemosterilant.

It is suggested from the data of these and other antifertility tests (Crystal, 1963) and the data of the present study that the influence of the number of reactive groups and of the nature of the molecular carrier moiety are interdependent. With respect to chemosterilization, it was observed (Crystal, 1963) that when the chemical was applied topically, compounds bearing three functional reactive groups resulted in more reliable induction of sterility than bifunctional compounds. These earlier studies also revealed that compounds with an equal number of functional groups but with different carrier moieties exerted variable antifertility effects ranging from complete sterility to fertility nearly 75% of normal.

The greatest inhibition of ovarian growth in the present study resulted from treatment with the benzoquinone derivative, a bifunctional compound. This result suggests that the quinone-ring system may have protein-associating properties if this compound is applied when nurse cells are undergoing endomitosis, and that these properties may be involved in further reaction after the molecule has been anchored to the genetic material by the alkylating aziridinyl groups. Of the two other compounds tested at 0.5%, tretamine was a more effective inhibitor of ovarian growth than thiotepa in newly emerged flies, but in 24-hour-old flies effects of both compounds were similar. Both are trifunctional but differ considerably in their carrier structures. The influence of the carrier moiety may be less important than the number of reactive alkylating groups in polyfunctional compounds such as thiotepa and tretamine. Methyl tretamine was obviously the least efficient compound of the four tested in its ability to inhibit ovarian growth. Production of similar chemosterilant and ovarian inhibitory effects required four times as much methyl tretamine as any of the other compounds. It has been previously concluded (Crystal, 1963) that the sterilizing efficacy of methylaziridinyl compounds, such as methyl tretamine, is less than that of the unsubstituted parent compounds, such as tretamine, from which they are derived.

The wide range of values obtained when measuring the volume of ovaries has been mentioned earlier in this report as an indication of the variability within groups. In general, measurements of ovaries of flies treated at 24 hours of age had a wider range of values than those of flies treated at either 0 to 4 hours of age or of untreated flies. This wider range perhaps indicated differences in physiological age as distinct from chronological age. The test insects within each series were maintained under identical conditions of environment. Nevertheless, it is probable that the rate of development varied sufficiently among individuals so that some flies were physiologically less, and some more, than 24 ± 2 hours old at the time of treatment. In this situation, treatment of flies at the terminal stages of endomitosis would inhibit ovarian growth to a greater extent than treatment after endomitosis had been completed. Greater variation in physiological development in 24-hour-old flies would be expected than in those less than 4 hours old because of the greater span of time available in which gaps in physiological age could be widened.

A study of Tables I and II reveals that flies with ovaries that had not attained a mean volume of 1.5 mm.^{3} were incapable of ovipositing. It was not until the ovaries of treated flies grew to more than 6 mm.^{3} in mean volume that at least 50% of the females laid eggs. However, there were no examples of greatly reduced fecundity among females that did oviposit. Antifertility effects in ovipositing females were expressed in the reduced hatchability of eggs as a result of the induction of dominant lethal mutations in the oocytes. No particular trend in these effects could be detected from the data in Table II. Thiotepa and apholate were somewhat more effective when newly emerged flies were treated; tretamine was ineffective at this age but completely effective when 24-hour-old flies were treated. Both the benzoquinone compound and methyl tretamine were highly effective in 24-hour-old flies, and induced large numbers of dominant lethal mutations. The data appear to justify the conclusion that the primary influence of aziridinyl compounds on the ovaries of 0- to 4-hour-old flies is the inhibition of oogenesis, and on those of 24-hour-old flies, the induction of mutations.

SUMMARY

Female screw-worm flies (*Cochliomyia hominivorax* (Coquerel)) 0–4 hours old or 24 ± 2 hours old were each treated topically by application to the dorsal thorax of 2 microliters of a solution of one of five aziridinyl chemosterilants. At 6 days of age, some mated females were given the opportunity to lay eggs and others were killed for measurements of the ovaries. The greatest inhibition of ovarian growth occurred during the endomitotic phase of the nurse cells (0–4 hours) and resulted in complete, or nearly complete, infecundity. The growth of ovaries of 24-hour-old females was but slightly or moderately affected, and the fecundity of such females was correspondingly greater than that of newly emerged females. However, the induction of many dominant lethal mutations in 24-hour-old flies greatly reduced or eliminated the fertility of such flies with resultant sexual sterility. It was concluded that the primary influence of aziridinyl compounds on the ovaries of 0- to 4-hour-old screw-worm flies is the inhibition of oogenesis and on those of 24-hour-old flies, the induction of mutations.

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THE MODIFICATION OF REPRODUCTION IN INSECTS TREATED WITH ALKYLATING AGENTS. II. DIFFERENTIAL SENSITIVITY OF OOCYTE MEIOTIC STAGES TO THE INDUCTION OF DOMINANT LETHALS

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Differential sensitivity among cell stages is commonly encountered in radiobiological studies. In investigations of chemical mutagens, similar differences in sensitivity are to be expected if these agents are truly radiomimetic. In the experiments reported herein, a study of the induction of dominant lethal mutations in the female germ cells of the screw-worm fly (*Cochliomyia hominivorax* (Coquerel); Diptera, Calliphoridae) was conducted to determine whether the sensitivity pattern was the same or similar for alkylating agents and gamma radiation. Comparisons of mutagenic efficiency were also made between a bifunctional and two trifunctional alkylating agents and between the two trifunctional agents having the same number and kind of functional groups but differing in the nature of the prosthetic group. Monofunctional alkylating agents are weak mutagens (Fahmy and Fahmy, 1956, 1958), and are relatively inefficient in the sterilization of insects (Crystal, 1963) and the induction of chromosome breaks (Alexander, 1960).

The present studies were also intended to supplement the rapidly accumulating amount of information on the production of sterility in insects by chemical means (LaBrecque, 1961; Knipling, 1962; Crystal, 1963). It is of interest to study the effects of chemosterilants on formed oocytes, since sterility need not be the result of reduced egg production but may be attained by dominant lethality in the zygotes. It has been shown that a chemical agent that produces infecundity in flies treated at a stage in which only immature germ cells are present may not be able to inhibit fecundity at a later stage; in such a situation sterility must then be the result of inviability of the eggs (see Crystal and LaChance, 1963). Finally, it is not enough to determine whether a chemical is mutagenic or not; quantitative data are needed to provide a basis for comparison of effectiveness between different sterilizing agents.

Many different types of compounds have been studied for their effects on the hereditary material of male *Drosophila* (Fahmy and Fahmy, 1956; Purdom, 1960) and of mice (Bateman, 1960; Moutschen, 1961). To date, the evidence is overwhelming that many of these agents are capable of inducing changes in the hereditary material very similar to radiation-induced changes (*i.e.,* point mutations, deficiencies, recessive lethals, dominant lethals, and chromosome breaks). Nevertheless, there is also evidence that the position, proportion, and time of induction of these primary genetic changes are different for radiation and chemical mutagens (Fahmy and Fahmy, 1956, 1960). Chemical mutagens themselves differ greatly with respect to their mutagenicity (Fahmy and Fahmy, 1956). With regard to varying sensitivity of female germ cells, Löbbecke and von Borstel (1962) have shown that when *Habrobracon* females are treated with nitrogen mustard or ethyl methanesulfonate, the oocytes in first metaphase are much more sensitive than oocytes in prophase I. To our knowledge, the three alkylating agents discussed in this report have not been studied for their effects on the induction of dominant lethals in meiotic oocytes of insects.

Materials and Methods

Adult females of the screw-worm fly are ideal for testing the effects of mutagens on oocytes in different stages of maturation. The female reproductive system of this insect contains two ovaries, each of which consists of 100–150 ovarioles, and development of the egg in each of these ovarioles occurs synchronously. By treatment of a single female, it is thus possible to treat hundreds of oocytes all in the same stage of development. Gravid females deposit as many as 200–250 eggs in a single egg mass. Oogenesis in this species has been described (LaChance and Bruns, 1963), and the different stages of meiosis, correlated with the age of the female, have been fairly well defined (LaChance and Leverich, 1962). From these previous studies it is known that 3-day-old females reared at 80° F. contain oocytes each with the nucleus in early prophase of the first meiotic division, that 4-day-old females contain almost fully mature eggs each with the oocyte nucleus in metaphase I, and that 5-day-old females contain a large number of fully mature oocytes in which meiosis has progressed up to anaphase I. Further growth and changes in the egg and its nucleus are arrested in this stage until the egg is laid.

Since the results of the preliminary experiments in this study were somewhat variable (compare experiments 3 and 4, Table I), it was decided to further standardize the treatment procedure to eliminate as many variables as possible. Although the synchronous development of many oocytes within a single female makes the screw-worm fly highly suitable for this kind of investigation, any factor that retards or accelerates the physiological aging of the female also affects the state of maturation of her oocytes. Thus, although within a given female all oocytes in the first egg chamber are in the same stage of development, variation among females of the same age will sometimes be encountered, due to many unknown factors that influence physiological age. Therefore, for experiments 5–14 reported in Table I, the testing procedure was slightly modified and standardized to minimize the introduction of extrinsic variables due to handling, crowding, anesthesia, and preparation of the chemical solutions. In these experiments the general procedure adopted was: (1) Flies were allowed to emerge from the puparia for a period of four hours, after which they were sexed. Emergence of the adults from the puparia was not retarded by storing the pupae at low temperatures (a common practice), since this might have affected the physiological age of the females. Thus, all of the females in a given group were within ± two hours in age. (2) The insects were maintained in an 80° F. colony room throughout the experiments. On the morning of the third day after emergence a solution of the chemical was prepared in an organic solvent and a given quantity was applied topically with a microapplicator to the dorsal thorax of one group of anesthetized females. This procedure was repeated on the fourth and fifth day after emergence with the same amount of freshly dissolved chemical applied topically to two other groups of females. Approximately 50 females were treated in each group. (3) All batches of the chemical to be used on the three successive days were weighed out by the same person on the same day. The solvent was added just before the chemical was applied, and, although a very small amount of solution was used in each test, a final volume of 10 ml. was made up to reduce errors in weighing and dilution. The chemicals were stored at freezing temperatures, yet at times they polymerized. When there was any evidence of polymerization, the chemical was recrystallized from carbon tetrachloride just before use. (4) Anesthesia prior to treatment of the females was accomplished by chilling for a very brief period rather than by knock-down with CO_2. (5) Untreated males were added to the cages of treated females approximately 16 hours before the females were egged instead of immediately after treatment. This procedure greatly reduced the chances of males becoming contaminated from chemical still on the thorax of the females or of sperm being exposed to the chemical within the spermathecae of the females. Studies on the metabolism of a related alkylating agent (metepa) indicated that

TABLE I

The induction of dominant lethal mutations in meiotic oocytes of Cochliomyia hominivorax by three alkylating agents

Females treated topically with the chemicals at 3, 4, or 5 days containing oocytes in early prophase, metaphase, or anaphase, respectively, subsequently mated with untreated males, and egged at 6 days. Assume dominant lethals equal 100 minus % corrected egg hatch.

Experiment number	Chemical	Amount applied (μl.)	Concentration		Total number of eggs scored	Hatchability of eggs (as percentage of controls) from females treated at		
			Per cent	$M \times 10^{-3}$		3 days	4 days	5 days
1	Tretamine	2.4	0.5	24.48	8,858	0.66	1.13	0.27
2		2.4	0.1	4.89	7,456	0.41	0.21	0.16
3*		2.4	0.01	0.489	9,585	31.39	26.0	3.50
4		2.4	0.01	0.489	9,917	90.77	37.64	15.24
5		2.4	0.01	0.489	9,145	96.39	50.08	1.99
6**		2.4	0.01	0.489	8,782	76.57	75.59	1.41
7		2.4	0.01	0.489	14,073	100.7	19.69	12.04***
8		2.0	0.01	0.489	10,738	100.4	33.69	3.44
9	Benzoquinone derivative	2.0	0.01	0.295	6,444	91.0	87.5	92.5
10		2.0	0.1	2.95	6,298	45.6	8.2	0.0
11		2.0	0.1	2.95	6,306	49.0	8.0	0.0
12	Thiotepa	2.0	0.01	0.529	6,137	93.5	78.6	67.7
13		2.0	0.01	0.529	6,281	117.1	79.2	60.8
14		2.0	0.05	2.65	6,098	100.5	27.6	1.2

* Pupae kept at 60° F. to retard emergence.

** High mortality, even among controls, attributed to overcrowding; control hatchability was low, and suggested that females were younger physiologically than chronologically at four days.

*** Females treated at 5 days egged at 8 days.

house flies, mosquitoes, stable flies, and screw-worm flies absorbed and metabolized the topically applied chemical very quickly (Plapp *et al.*, 1962; Chamberlain, unpublished). (6) When the females were 6 days old, oviposition was induced by presenting each female with a small piece of lean meat and keeping her at a temperature of 90–96° F. for a few hours. At the low concentrations of chemicals used in these experiments, toxicity was not encountered, and the females deposited the normal numbers of eggs.

To insure that eggs deposited by unfertilized females were not included in any egg sample used for hatchability studies, each female was allowed to oviposit individually. After producing an egg mass the female was sacrificed and the spermathecae examined for the presence of sperm. Only egg masses from inseminated females were used. Egg masses were separated and plated for hatchability studies according to the method described by LaChance and Leverich (1962). All hatchability figures were based on counts of approximately 2000 eggs for each dose and age group. Hatchability of the eggs was scored after incubation for 28–30 hours at 80° F. After correction for the number of natural deaths in the controls, it was assumed that all unhatched eggs were the result of a dominant lethal change induced in the oocyte of the treated female. Sex-linked recessive lethal changes would also have contributed to death in the F_1 male embryos; but insemination by normal, untreated males prevented detection of autosomal recessive lethal changes, and hatchability tests cannot detect dominant lethal changes expressed beyond the egg stage.

The experiments reported in this paper were performed over a period of 7 months, during which 16 different sets of controls were examined. Control hatchability ranged from 66% to 95%. The data in Table I have been corrected for the respective controls. A total of 126,886 eggs from treated and control females was examined for embryonic dominant lethals.

The three chemicals used in these tests were: (a) 2,4,6-tris(1-aziridinyl)-*s*-triazine(= tretamine = TEM = triethylenemelamine); (b) 2,5-bis(1-aziridinyl)-3,6-bis(2-methoxyethoxy)-*p*-benzoquinone; and (c) tris(1-aziridinyl)phosphine sulfide (= thiotepa = thio-TEPA = triethylenethiophosphoramide). These chemicals will hereafter be referred to as tretamine, benzoquinone, and thiotepa. All three are alkylating agents. Both tretamine and thiotepa have three aziridinyl alkylating groups but differ in that in tretamine these three groups are carried on a triazine ring, whereas in thiotepa the three groups are attached to phosphine sulfide. The benzoquinone compound has only two aziridinyl alkylating groups attached to the quinone ring in the 2,5 positions, but also carries two methoxyethoxy chains in the 3,6 positions.

RESULTS

The first chemical to be studied in this series was tretamine. Results of the first two experiments performed (experiments 1 and 2 in Table I) indicated that concentrations of 0.5% and 0.1% were highly mutagenic to all oocyte stages, inducing nearly 100% dominant lethals. At 0.01% (Table I) some viable eggs were laid. This finding indicated that a level had been reached at which differential sensitivity of the meiotic stages could be investigated.

The experiment was repeated five times with 2.4 μl. of tretamine applied at a concentration of 0.01% and the results are summarized in Table I (experiments

3–7). At this concentration, great differences among the meiotic stages occurred in their sensitivity to the chemosterilant. In all experiments, the oocytes in prophase I were very resistant to tretamine, whereas the oocytes in anaphase I (5-day-old females) had large numbers of dominant lethals. In general, high rates of dominant lethals were also induced in oocytes treated in metaphase I (4-day-old females), but the sensitivity of this stage seemed to be more variable. The reasons for the variability of the metaphase I stage will be discussed below.

The data in Table I establish a clear trend in oocyte sensitivity to tretamine, from resistance in early prophase I to high sensitivity in anaphase I. Only in experiment 3 were a moderate number of dominant lethals induced in early prophase I oocytes. In this series, retardation of emergence of the adults by chilling (not as carefully avoided as in later trials) could have altered the physiological age of the females and consequently the speed of maturation of the oocytes; thus, although 3-day-old females were treated, oocyte maturation had perhaps progressed further than in other groups of comparable chronological age. The equal numbers of dominant lethals induced in 3- and 4-day-old females in experiment 6 can, to some extent, be attributed to the very crowded cages used in this experiment; these conditions perhaps delayed aging and maturation in the females so that the sensitive period was not reached until the females were more than four days old.

In order to further test the hypothesis that the stages in oocyte maturation differ in sensitivity to the induction of dominant lethals by chemical mutagens, similar experiments were conducted with thiotepa and benzoquinone. The results of these experiments are presented in Table I. In general, they agree remarkably well with the results obtained with tretamine. At the higher concentrations, early prophase I oocytes were extremely resistant to both of these chemicals, and metaphase and anaphase I were very sensitive. Neither of these chemicals was very effective in inducing dominant lethals at a concentration of 0.01%, but thiotepa was definitely more damaging than benzoquinone. Thiotepa was very mutagenic to anaphase I oocytes at 0.05%. This intermediate concentration was not tested with benzoquinone because the earlier test at 0.01% indicated low mutagenic efficiency of the compound. However, at a concentration of 0.1% dominant lethals were induced in half of the prophase I oocytes, whereas 92% and 100% dominant lethals were induced in the other two stages. Thus, these three chemicals produced different amounts of genetic damage when equal concentrations were tested. In descending order of mutagenic efficiency, tretamine was first, thiotepa second, and benzoquinone third.

In order to assess the role of molecular structure in mutagenic activity, it was thought desirable to compare the activity of different compounds on the basis of molar concentration. The data on chemical concentrations in Table I have been expressed in both per cent concentration and molarity. A comparison of the compounds indicates that tretamine was the most effective mutagen of the three. However, on the basis of molarity, benzoquinone appears to be at least as potent a mutagen as thiotepa, but further tests are needed to clarify this point.

Before a meiotic stage can be assumed to represent a stage of high sensitivity, one must rule out the possibility that one stage has had more time to recover from the treatment than another before having to cope with the crises of the meiotic divisions, syngamy and embryogenesis, which follow fertilization of the egg. Such a possibility must be ruled out especially with regard to results obtained with 3-day-old females, which were allowed three days for recovery between treatment and egging in contrast to 5-day-old females, which had only one day to recover. In order to study this possibility, experiment 7 was performed in a manner slightly different from the others. In experiment 7 the females treated at three or four days of age were egged at 6 days, and those treated at 5 days were egged at 8 days. The results showed a high incidence of dominant lethals induced in 5-day-old females, but a virtual absence of lethals in females treated at three days of age. Thus, the time elapsing between treatment and oviposition did not alter the results. Also, in *Habrobracon* oocytes there is no evidence for recovery from nuclear damage induced by nitrogen mustard (von Borstel, 1955). It can therefore be concluded that the meiotic stage of the oocyte nucleus at the time of treatment does condition the amount of dominant lethality induced in the oocytes. Similar results have been obtained with gamma radiation (LaChance and Leverich, 1962).

Discussion

Treatment of various meiotic stages in the oocytes of screw-worm flies has demonstrated consistent trends in sensitivity to three chemical mutagens. Although some variation existed in the different replicates, especially with treatment of 4-day-old females (Table I), these variations did not obscure the major trend demonstrated, the progression from resistance to susceptibility to chemical mutagens during maturation of the oocytes. It must be emphasized that varying sensitivity of the metaphase I stage was encountered only with treatments at the borderline of effectiveness. At more damaging concentrations (those that resulted in 100% dominant lethals in anaphase I oocytes), the metaphase I oocytes also exhibited high sensitivity (Table I, experiments 7, 8, 10, 11, 14).

The present data, as well as those obtained in tests with gamma radiation (LaChance and Leverich, 1962), show that during the period between three and four days in the adult life of screw-worm females, the oocytes are changing from resistance to sensitivity to both types of mutagens. However, the energy from gamma radiation can be delivered and effect damaging results in a matter of minutes, but chemical mutagens probably induce genetic damage over a longer period, during which the oocytes could be continuing their transition from resistant to sensitive stages. This extended period of activity would, of course, result in some variability, which would be more evident at the lower concentrations.

The chronological age of the females were controlled to ± two hours, but it is the physiological age of the female that determines the progress of development in the oocytes. Even within closely timed groups, some females may be more advanced in development than others. Because of the transitional nature of the oocytes between three and four days, perhaps occasionally some 4-day-old females were treated before the oocytes entered the period of sensitivity. The data obtained by sampling approximately 2000 eggs from many females were representative of an average of the stages in oocyte development present at the time of treatment. Thus, data taken when the females were four days old should not be interpreted as representing a period of intermediate sensitivity but, rather, one of a transitional nature, in which most but not all of the females had reached the sensitive stage in oocyte development. This conclusion is supported by unpublished cytological observations on ovarioles dissected from females of this age group.

Although the cytological difference between metaphase and anaphase I is very slight, there seemed to be a constant difference between them in response to chemical mutagens. It was surprising to find that 4-day-old oocytes were somewhat less sensitive than 5-day-old oocytes; in radiation experiments the reverse has been consistently true (LaChance and Leverich, 1962). Perhaps this response is indicative of some basic difference in the mode of action between the two types of mutagens.

It was of interest to note that concentrations of tretamine as low as 0.5% completely inhibited ovarian growth when administered to newly emerged females, but when administered when the flies were 24 hours old, had little effect on ovarian growth (Crystal and LaChance, 1963). However, although a normal number of eggs was produced after treatment of 3–5-day-old females, more than 99% contained dominant lethals. These findings illustrate the varied and powerful effects of this mutagen on insect reproduction.

The data indicate that tretamine is a more potent mutagen than thiotepa. Since both of these compounds have three aziridinyl groups that react with nucleoproteins by alkylation, the difference in activity between them serves to reemphasize the role of the prosthetic group in mutagenicity (Fahmy and Fahmy, 1956, 1960) and in chemosterilization (Crystal, 1963). The benzoquinone compound is bifunctional and, although it was less effective than thiotepa at a concentration of 0.01%, the higher concentration (0.1%) gave definite evidence of being mutagenic, even to resistant prophase I oocytes. The evidence is sufficient to classify these three chemicals as fairly powerful alkylating agents that are radiomimetic, at least to the extent of showing the same pattern of effect on developing oocytes. The basic cause for the greater sensitivity of some meiotic stages to mutagens cannot yet be ascertained, no more than it can definitely be stated why these same stages are more sensitive to radiation (see Whiting, 1945; Bozeman and Metz, 1949; Sparrow, 1951; LaChance and Leverich, 1962).

It is fairly certain that dominant lethals are a manifestation of chromosome breakage in the meiotic oocytes (von Borstel, 1955). Fahmy and Fahmy (1954) have shown that dominant lethals induced in *Drosophila* sperm by tretamine are due to primary breaks that undergo noneucentric rearrangements. The end result of such chromosome aberrations in oocytes would be lethality in the zygotes due to failure of completion of the meiotic division, or chromosome loss or imbalance in the embryo. Whether the meiotic stages differ in the amount of genetic damage initially induced or in the degree of recovery or repair of the genetic damage is still unknown. Possibly permeability to chemicals differs in the various stages due to changes involving the nuclear membrane. Also the varying ability of broken chromosomes to rejoin after treatment of different meiotic stages has been offered as a plausible explanation for differences in radiosensitivity (Whiting, 1945). To date no better hypothesis has been put forth, and this explanation may therefore be equally tenable for radiomimetic substances, providing that the chromosome breaks are induced in fully formed chromosomes shortly after treatment and not at some later stage, such as during chromosome replication for cleavage divisions in the zygote. Clearly the basis for the differential sensitivity of meiotic stages to alkylating agents depends on whether these chromosome breaks become actual immediately or at some later stage of development in the egg.

The results are likely to be misleading if dominant lethals are assessed on the basis of inviability alone without subsequent cytological analysis (Fahmy and Fahmy, 1958; Purdom, 1960). This is an important point when adult males are treated and subsequently mated to untreated females, since inviability in such a situation could be due to aspermia. However, inviability in our experiments was certainly due to dominant lethality, since females were treated at stages in which there is no germinal selection against damaged oocytes and mated to untreated males. All eggs studied were deposited by females that had been examined for the presence of motile sperm.

A comparison of mutagenic efficiency between chemical mutagens and ionizing radiation is highly speculative. The 50% dominant lethal dose for *Cochliomyia* oocytes irradiated in early prophase I is 7,939 r (LaChance and Leverich, 1962); about 60% dominant lethals were induced by 10,000 r. Thus, treatment with 2.0 μl. of 0.1% benzoquinone appears to be about as mutagenic as 7,939 r, and 2.4 μl. of 0.1% tretamine (4.89×10^{-3} M) is vastly more damaging to resistant oocytes.

The present data are consonant with those of Löbbecke and von Borstel (1962) in which the metaphase I oocytes of *Habrobracon* were found vastly more sensitive than prophase I oocytes to treatment with nitrogen mustard and ethyl methanesulfonate when both dominant and recessive lethals were studied.

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SUMMARY

When adult females of the screw-worm fly, *Cochliomyia hominivorax,* are topically treated with alkylating agents, dominant lethal mutations are induced in the oocytes. Since the meiotic stage of the oocytes is correlated with the age of the female, it is possible to treat oocytes in either early prophase, metaphase, or anaphase of the first meiotic division. When such treatment is made, fewer dominant lethals are induced in prophase oocytes than in oocytes in the other two stages. This trend was consistently found in studies with three chemical mutagens: (a) tretamine (2,4,6,-tris(1-aziridinyl)-*s*-triazine), (b) a benzoquinone derivative (2,5,-bis(1-aziridinyl)-3,6,-bis(2-methoxyethoxy)-*p*-benzoquinone), and (c) thiotepa(tris(1-aziridinyl)phosphine sulfide). In addition to demonstrating the differential sensitivity of various oocyte meiotic stages to these agents, this report compares the mutagenic efficiency of the agents and discusses the possible basis for differential sensitivity of cell stages to mutagens.

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STUDIES ON HOLOTHURIAN COELOMOCYTES. I. A SURVEY OF COELOMOCYTE TYPES

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The following 24 reports deal entirely or in part with the description of cellular elements in the coelomic fluid of holothurians: Becher (1907); Boolootian (1962); Boolootian and Giese (1958); Crescitelli (1945); Cuénot (1891); Dawson (1933); Dendy (1898); Endean (1958); Gerould (1896); Haanen (1914); Hamann (1883); Hérouard (1889); Hogben and van der Lingen (1928); Howell (1885); Jourdan (1883); Kawamoto (1927); Kindred (1924); Knoll (1893); Kollmann (1908); Ohuye (1936, 1938); Semper (1868); Théel (1920); Van der Heyde (1922). Although great variation and confusion exists among these descriptions, at least nine basic categories of holothurian coelomocytes appear to have been described to date:

1. Nucleated cells containing red pigment, generally believed to be hemoglobin, and one or more yellow refractile granules. Kindred (1924) and Hyman (1955) refer to these cells as hemocytes, the name adopted here.
2. Amoeboid cells with homogeneous cytoplasm or cytoplasm containing a variety of granules and vacuoles and with bladder-like, petaloid, or filiform pseudopodia.
3. Weakly amoeboid cells partially or completely filled with colorless spherules called morula cells by Endean (1958).
4. Small spherical to oval cells that may bear one to three filiform pseudopodia have been described by three authors, Jourdan (1883), Ohuye (1936), and Endean (1958). This category of coelomocytes will be called lymphocytes in the present report.
5. Coelomocytes called crystal cells containing large crystalline bodies of various shapes.
6. Small spherical bodies devoid of a nucleus, but occasionally containing a colored granule, called minute corpuscles by Kawamoto (1927) and Ohuye (1936).
7. Masses of yellow-brown granules have occasionally been reported in the coelomic fluid of holothurians. These aggregations will be called brown bodies in the present report.
8. Flagellated cells called vibratile cells were reported by Kindred (1924), Boolootian and Giese (1958), and by Boolootian (1962).
9. Highly refractile fusiform cytoplasmic bodies called fusiform cells.

To test the validity of this system of classification of holothurian coelomocytes, and to determine how uniformly these cell types may be distributed among holothurians, the author completed a survey of the coelomocytes of eleven species of holothurians, representing four orders and five families.

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MATERIALS AND METHODS

The coelomocytes of the following eleven species of holothurians, common in Puget Sound and the San Juan Archipelago, were surveyed and described:

Order Dendrochirota: *Cucumaria lubrica, C. miniata, C. piperata, Eupentacta pseudoquinquesemita, E. quinquesemita, Pentamera populifera, Psolidium bullatum, Psolus chitonoides*
Order Aspidochirota: *Parastichopus californicus*
Order Molpadonia: *Molpadia intermedia*
Order Apoda: *Leptosynapta clarki*

Direct observations of coelomic fluid were made under oil immersion, using both ordinary light microscopy and phase contrast microscopy. Samples of coelomic fluid were withdrawn from the perivisceral coelom with a 10-ml. hypodermic syringe fitted with a #27 stainless steel needle. Whenever possible, the sample was taken from the region of the introvert of a fully extended holothurian. Commonly, holothurians would contract partially during the sampling process. As the contraction occurred, the coelomic fluid was automatically forced into the syringe.

Changes in the morphology of the coelomocytes, presumably due to contact with glass and metal surfaces, were kept to a minimum by repeated previous exposures of the inner surfaces of the syringe and needle, and the surfaces of the slides and coverslips to coelomic fluid of the same species of holothurian. Apparently these exposed surfaces were covered by an invisible coating of protein or other material derived from the coelomocytes or coelomic fluid. A different syringe and needle were used for each species studied and were cleaned by rinsing them with distilled water and filtered sea water. No attempt was made to seal the preparations to prevent drying. Fresh mounts without seals remained in excellent condition for 30 to 60 minutes and often a single preparation remained usable for several hours. Because of the ease with which fresh preparations were obtained, new samples could be taken at frequent intervals.

Coelomocytes were vitally stained using a modification of the technique developed by Dawson (1933). Slides were prepared with 0.025% solution (w/v) of the vital dye in absolute alcohol, instead of saturated solutions of the dye in alcohol as used by Dawson.

Coelomocytes were vitally stained with solutions of the following dyes: neutral red, Janus green B, brilliant cresyl blue, methylene blue, Bismarck brown, and toluidine blue. Optimum staining of the coelomocytes occurred within 15 minutes.

Permanent mounts of coelomocytes were made as follows: Wet smears of coelomic fluid were fixed with osmium or formalin vapors for 30 minutes. Following fixation the smears were dried to insure adherence of the cells to the surfaces. Dried smears were dipped in distilled water to remove adhering salt crystals before staining and mounting. Erhlich's hematoxylin and eosin were used in routine staining. Smears of coelomic fluid of species possessing hemocytes were also stained according to the technique of Ralph (1941) for hemoglobin detection.

Samples of coelomic fluid from the water vascular system were taken from a tentacle or from the polian vesicle in dissected specimens. To determine the varieties of coelomocytes present in the hyponeural sinus and the hemal system of holothurians, sections of the digestive tract and body wall were prepared. The tissues were fixed in Bouin's fixative, dehydrated in an ethyl alcohol series, sectioned at 10 microns, and stained with Erhlich's hematoxylin and eosin.

RESULTS

Of the nine types of holothurian coelomocytes recognized in the literature, only six (hemocytes, amoebocytes, morula cells, lymphocytes, crystal cells, and brown bodies) were found to be normally present in the coelomic cavities of the eleven species of holothurians investigated by the author. The three remaining coelomocyte types (minute corpuscles, fusiform cells, and vibratile cells) appear to be either artifacts or cells foreign to the coelomic cavities in which they were found. Any one of the coelomocyte types found in a specific species was found in all of the coelomic compartments with the exception of the hyponeural system in which no coelomocytes were observed. The relative members of coelomocyte types appear to vary from one coelomic compartment to another within a given specimen.

1. Hemocytes (Plate I; Fig. A)

Hemocytes were present in the coelomic fluid of seven of the eleven holothurian species investigated: *Cucumaria lubrica, C. miniata, C. piperata, Eupentacta pseudoquinquesemita, E. quinquesemita, Molpadia intermedia,* and *Pentamera populifera.* Hemocytes were abundant enough to impart a scarlet color to the fluids of the perivisceral coelom and the water vascular system of *C. miniata, C. piperata* and *Molpadia intermedia,* and to the fluid of the water vascular system but not the perivisceral fluid of *C. lubrica.* The perivisceral coelomic fluid of *C. lubrica* and the fluid of the water vascular system of *C. lubrica, E. pseudoquinquesemita,* and *E. quinquesemita* were pink due to the presence of hemocytes. In all other cases where hemocytes were present, they were too few in number to impart color to the coelomic fluid.

Isolated hemocytes, or hemocytes in small clusters, appeared straw-yellow in color when viewed microscopically. The shape and size of holothurian hemocytes varied slightly even within a single species. The hemocytes of the Dendrochirota varied from flattened or oval biconvex discs to spherical cells. Disc-shaped cells measured 4 to 6 microns in thickness; the faces of oval cells measured 12 to 16 microns along the shorter axis and 13 to 23 microns along the longer axis. Spherical hemocytes measured from 9 to 11 microns in diameter.

The hemocytes of the Dendrochirota were generally smooth in outline, but occasionally a small filiform pseudopodium, seldom more than 5 microns in length, was formed. Hemocytes in drying preparations produced bleb-like cytoplasmic protuberances measuring up to 5 microns in length. The blebs eventually pinched off from the hemocytes and appeared in the coelomic fluid as small structureless spherules similar to minute corpuscles. A single hemocyte occasionally produced several of these cytoplasmic spherules and eventually the entire cell cytolyzed.

The hemocytes of the Dendrochirota studied possessed a thin hyaline ectoplasm and a pigmented endoplasm in which were located the following cytoplasmic inclusions: (1) one, infrequently two, excentrically placed nuclei. Disc-shaped or oval biconvex hemocytes had nuclei that were disc-shaped, approximately two microns

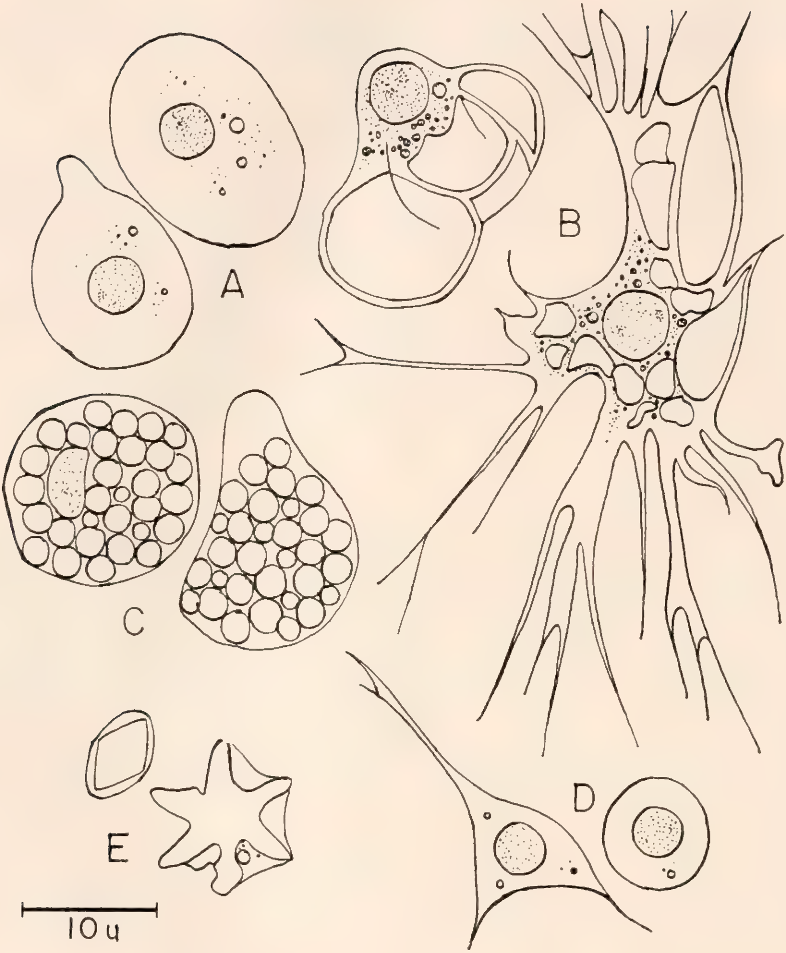

Plate I

A. Two hemocytes of *Cucumaria miniata.* The lower left cell is in the process of producing a bleb of cytoplasm that would later be pinched off as a minute corpuscle.

B. Two amoebocytes of *Cucumaria miniata.* The upper left cell is in the bladder or petaloid stage; the amoebocyte to the right is in the filiform stage.

C. Two morula cells (trephocytes) of *Cucumaria miniata.* The cell on the left shows the nucleus.

D. Two lymphocytes of *Cucumaria miniata.*

E. Two crystal cells: a rhomboidal crystal cell from *Eupentacta quinquesemita* and an imperfect star-shaped crystal from *Psolus chitonoides.*

thick and 4 to 6 microns in diameter. Spherical hemocytes had spherical nuclei approximately 4 microns in diameter. All nuclei appeared coarsely granular. In favorable preparations stained with methylene blue, one to three minute bodies interpreted as nucleoli were observed.

(2) Several small Janus green B-positive granules were located near the nuclei. Occasionally these granules appeared to be in actual contact with the nuclear membrane.

(3) One to six yellow refractile granules, 1 to 3 microns in diameter, were characteristic of all but a few hemocytes found in dendrochirotes. Yellow refractile granules appeared to intergrade with the Janus green B-positive granules described above, and may possibly be derived from them. Among the granules possessing characteristics intermediate between the Janus green granules and the yellow refractile granules were the following: (a) Larger than average Janus green granules containing a clear vacuole; (b) still larger Janus green granules with vacuoles containing a dense, slightly yellow material; and (c) large yellow refractile granules possessing an enclosing membrane stainable with Janus green B.

The inclusion of a yellow refractile granule in blebs of cytoplasm produced during the cytolysis of a hemocyte was not uncommon.

The hemocytes of *Molpadia intermedia* differed in size and shape from the hemocytes of the Dendrochirota. The hemocytes of *Molpadia* were rod- or cigar-shaped, measuring 20 to 33 microns in length and 4 to 7 microns in diameter. The hemocytes of *Molpadia* often were bent on their long axis forming L-, J-, or V-shaped cells. Occasionally, but not commonly, oval or disc-shaped hemocytes, similar to those of the Dendrochirota, were observed in *Molpadia.* Each cell contained a single spherical, excentrically placed nucleus measuring approximately 4 microns in diameter. The nuclei often appeared to fit tightly against the cell membrane of the rod-shaped hemocytes. One or two yellow refractile granules were also characteristic of the hemocytes of *Molpadia.*

A hyaline ectoplasm was not visible in the hemocytes of *Molpadia,* but when preparations were allowed to stand, thin ectoplasmic filaments were extended from the cells. Hemocytes of *Molpadia* cytolyzed sooner than those of the Dendrochirota.

The hemocytes of all seven species of holothurians stained a deep brown when subjected to Ralph's technique for the detection of hemoglobin. This color change is considered a positive indication that hemocytes contain hemoglobin.

2. *Amoebocytes (Plate I; Fig. B)*

Amoebocytes were observed in the hemal systems and in all the coelomic cavities, with the exception of the hyponeural systems, in all eleven species of holothurians studied. With the exception of hemocytes in *Cucumaria miniata* and *Molpadia,* amoebocytes are apparently the most abundant coelomocyte type in any species studied.

Amoebocytes changed form during the period of even brief observations. The changes involved the form of the pseudopodia, redistributions of the cellular inclusions, and even affected the form of the nucleus. Consequently, difficulty is encountered in attempting a general description of holothurian amoebocytes. However, the behavior of the amoebocytes of the eleven species of holothurians investigated was remarkably similar.

All amoebocytes possessed clear hyaline ectoplasm that produced pseudopodia and a granular endoplasm containing the single nucleus and various cytoplasmic inclusions. The pseudopodia of holothurian amoebocytes were of two forms, petaloid or bladder-like, and filiform. Careful observations of fresh preparations revealed that all amoebocytes possessed petaloid or bladder-like ectoplasmic pseudopodia that radiated in any direction from the central endoplasmic mass of the cell. One to several such pseudopods existed at any one time. Each pseudopodium appeared to consist of a thin ectoplasmic layer of cytoplasm enclosing a clear, colorless bladder or vacuole. Amoebocytes with bladder-like or petaloid pseudopodia float freely in the coelomic fluid, slowly rolling over and over.

In fresh preparations, the petaloid pseudopodia appeared to collapse, and a redistribution of the cytoplasm produced branching filiform pseudopodia of considerable length, often intermeshing with the filiform pseudopodia of other amoebocytes. Petaloid amoebocytes were capable of direct transformation into amoebocytes with filiform pseudopodia, but the transformation of filiform pseudopodia into petaloid pseudopodia was never observed.

The granular endoplasmic mass of an amoebocyte measured from 6 to 13 microns in diameter; petaloid pseudopodia measured 5 to 15 microns in length; filiform pseudopodia were commonly up to 50 microns or more in length.

The nuclei of amoebocytes varied in shape depending on the configuration of the cell body. Spherical nuclei measured 4 to 5 microns in diameter; oval nuclei 4 to 5 microns along the long axis and 2 to 3 microns along the shorter axis. Bean-shaped to flattened nuclei were situated like caps in the endoplasmic mass of amoebocytes with all pseudopodia extended in one direction. The nuclei appeared granular; preparations stained with methylene blue revealed one or two structures interpreted as nucleoli.

The nuclei of amoebocytes were usually obscured by endoplasmic granules ranging from fine Janus green B-positive granules to yellow-brown refractile granules measuring as much as 2.5 microns in diameter, and by small vacuoles. All the granules in any one amoebocyte were not positively stained with any one vital dye, but all of the granules in any amoebocyte were stained by at least one of the vital dyes used.

Commonly, amoebocytes were observed to contain one or more pycnotic nuclei in addition to their own normal one; occasionally, whole hemocytes or other coelomocytes were observed within an amoebocyte. As many as three hemocytes were observed within a single amoebocyte of *Cucumaria miniata.*

3. Morula cells (Plate I; Fig. C)

Morula-like coelomocytes were found in the hemal system, perivisceral coelom, and the water vascular systems of the eleven species of holothurians investigated. Morula cells were observed in all connective tissue layers of sectioned material and were particularly abundant in the connective tissue of the dorsal mesentery in the region of its junction with the gut.

Morula cells were spherical cells ranging in diameter from 8 to 20 microns; they varied in size within a single species. The general proportion of morula cells to the entire number of coelomocytes varied among different individuals in any one species.

The clear hyaline cytoplasm of a morula cell contained colorless spherules measuring from 0.5 to 5.5 microns in diameter. The spherules of any one morula cell were uniform in size. The spherules completely filled the cell, obscuring the single oval to spherical nucleus. The nucleus measured from 3 to 5 microns in diameter.

Morula cells were capable of feeble amoeboid movements; a morula cell occasionally extruded a short, blunt hyaline pseudopodium, seldom over 4 microns in length. After the pseudopodium was extended the spherules flowed, rolling over one another, into the pseudopodium.

Morula cells in the coelomic fluid and mesenteries were intensely basophilic; preparations stained with toluidine blue showed each spherule to be composed of an inner core that stained pale blue and an outer shell that was metachromatic and stained pink to violet. The cytoplasm around the spherules stained a pale blue.

Morula cells present in the connective tissue layers of the body wall were eosinophilic rather than basophilic; morula cells in the hemal system, or closely associated with it, were amphiphilic.

Few cellular inclusions other than the spherules were observed in morula cells. Rarely a Janus green B-positive granule or a small yellow refractile granule was observed in the hyaline cytoplasm of the morula coelomocytes.

4. *Lymphocytes* (*Plate I; Fig. D*)

Lymphocytes were present in the fluid of the perivisceral coelom, the water vascular system, and the hemal system of all eleven species of holothurians studied. Lymphocytes in their basic form were spherical cells 6 to 8 microns in diameter with clear hyaline cytoplasm. A single, finely granular spherical nucleus, 3 to 6 microns in diameter, nearly filled the entire cell. One or two tiny nucleoli were occasionally visible. One or two Janus green B-positive granules were the only cytoplasmic inclusions. Two such granules present in the same cell were usually situated at opposite poles of the cell. Filiform pseudopodia up to 15 microns in length were characteristic of large lymphocytes. No pseudopodium, once formed, was ever observed to be retracted. The pseudopodia were located on the poles of the cells where the Janus green B-positive granules were located. Lymphocytes with one pseudopodium and a Janus green B-positive granule at its base were as common as lymphocytes with two pseudopodia at opposite poles of the cell. Occasionally triangular lymphocytes were observed with a filiform pseudopodium extending from each of the angles. A Janus green B-positive granule was usually present at the base of each of the three pseudopodia. The pseudopodia of lymphocytes were commonly branched. The branching pseudopodia of lymphocytes were never as complex as the branching filiform pseudopodia of amoebocytes.

5. *Crystal cells* (*Plate I; Fig. E*)

Coelomocytes containing crystals were observed in the fluids of the perivisceral coelom and water vascular system of all the holothurians studied with the exception of *Leptosynapta clarki*. Crystal cells were not observed in the fluids of the hemal system of any of the species.

The crystals were commonly rhomboidal; the size and proportions of the rhomboids varied greatly even within a single organism. Crystals ranged from tiny rhomboids, so small their form was recognized with difficulty, to rhomboids measuring 24 microns in length and 16 microns in width. A single rhomboidal crystal measuring 11 microns on a side was observed in *Eupentacta quinquesemita*. The rhomboidal crystals were, however, seldom more than three microns thick regardless of their other dimensions. The average rhomboidal crystal measured 5 to 6 microns on a side, 5 to 6 microns long, 4 to 5 microns wide and 1 to 2 microns thick.

The cells enclosing these crystals appeared only large enough to house the crystal. The thin layer of hyaline cytoplasm surrounding the crystal was visible only when viewed from a favorable angle. The nuclei of crystal-bearing cells were difficult to see. The single nucleus was often flattened and closely applied to the crystal. Occasionally, it was impossible to locate any nucleus in a crystal cell; whether one existed or not is open to question.

One or two Janus green B-positive granules were commonly observed in the clear cytoplasm surrounding the crystal. Occasionally a large yellow refractile granule, similar to those seen in hemocytes and amoebocytes, was observed in the cytoplasm of crystal-bearing coelomocytes. Not uncommonly two or three crystals were found within a single cell. The extra crystals were piled one on the other in such a manner that three superimposed crystals gave the appearance of a 6-pointed star. Solid 6-rayed star-shaped crystals, measuring 6 microns from point to point and 2.5 microns thick, were also observed. Rhomboidal crystals with knobs or spines borne on the acute angles were commonly observed. Imperfect rhomboids and irregular rhomboidal crystals were common.

In addition to cells containing typical rhomboidal and star-shaped crystals, *Psolus* and *Psolidium* possessed coelomocytes containing crystals larger and more elaborate in form. These crystals appeared as imperfect stars with one to six rays elongated into long spine-like processes. The spines measured up to 43 microns in length. Entire crystals occasionally measured over 70 microns in length. Often the basic star was aborted and appeared as an oval plate from which two, three, or four spines projected in the same plane.

The ends of the long spines were sometimes expanded into rhomboidal-shaped structures giving the appearance of a spear head. Surrounding each of these large crystalline structures was a single cell. The clear hyaline cytoplasm stretched web-like between adjacent spines. One or two yellow refractile granules or one or two fine Janus green-positive granules were often observed near the central mass of the crystal. The yellow refractile granules measured up to 4 microns in diameter.

Small irregular bits of crystalline material were rarely observed within vacuoles in small spherical cells. A thin layer of hyaline cytoplasm surrounded the vacuole; the nucleus and usually one yellow refractile granule were forced to one side of the cell by the vacuole.

No crystals were stained by any of the vital dyes used. Crystal cells were nearly impossible to recognize in fixed material because the crystals, which dissolved under the slightest osmotic stress, were lost during the staining and dehydration process. When the crystals dissolved, the coelomocytes containing them collapsed.

Crystal cells were the least abundant coelomocyte type in any species studied. In the case of *Cucumaria miniata* one crystal cell per slide preparation was usually the most one could expect to find. On several occasions, however, there appeared to be an abundance of crystal cells (up to five or ten) in a single preparation. Within a day, however, the number of crystal cells in the same animal was reduced.

6. *Minute corpuscles*

Spherical minute corpuscles, 1 to 6 microns in diameter, appeared in fresh preparations of coelomic fluid of all eleven species of holothurians investigated. The number of minute corpuscles increased as the preparations aged, due to the fragmentation of other coelomocytes; minute corpuscles apparently are enucleate fragments of cytolyzed amoebocytes or hemocytes. Occasionally a cytoplasmic granule was included in the spherical mass of the minute corpuscle. Minute corpuscles originating from hemocytes were differentiated from those originating from amoebocytes on the basis of the straw yellow color of the hemoglobin included in them. The production of minute corpuscles by the fragmentation of hemocytes and amoebocytes was easily observed.

7. *Fusiform cells*

Fusiform cells were occasionally observed in the coelomic fluid of all eleven species of holothurians investigated. Fusiform cells were structureless columns of cytoplasm tapering at either end. Only once was a nucleus seen in a fusiform cell. Several times one to three Janus green B-positive granules were observed in the cytoplasm. Usually, fusiform cells contained no visible inclusions. Fusiform cells ranged from 1 to 3 microns in diameter and up to 15 microns in length.

Fusiform cells were not present in every specimen of a given species. Their relative numbers increased with repeated handling and sampling from a given specimen.

8. *Brown bodies*

Brown bodies are aggregations of yellow to brown granular material accumulated in the coelomic cavities, hemal systems, and tissues of holothurians. All the holothurians in the present study possessed brown bodies. Masses of brown body granules accumulated in the anterior regions of the water vascular system and in the posterior regions of the perivisceral coelom among the suspensor muscles of the cloaca, and, to a lesser extent, in the walls of the respiratory trees. Brown bodies were also commonly observed in the walls of the tentacles of the dendrochirote and aspidochirote holothurians. Accumulations of brown bodies were also noted in this cavity of the blind, regenerating gut of *Parastichopus*. Masses of yellow to brown granules were also observed embedded in the gut and body wall tissues of holothurians.

Brown body material in *Leptosynapta clarki* accumulated in the stalked ciliated funnels and urns found along the junction of the mesenteries that support the gut and the body wall; in psolids brown body material was deposited between the peritoneum and the wall of the ventral sole in addition to the locations mentioned above.

The yellow to brown granules commonly observed in amoebocytes resemble granules of brown body material; spherical cells, approximately 10 microns in diameter and completely filled with yellow to brown granules 0.5 to 3 microns in diameter, were interpreted as amoebocytes filled with brown body granules. Smears of brown bodies revealed this material to be composed of degenerating cells filled with yellow granular material plus occasional morula cells and hemocytes presumably trapped in the aggregation. One brown body taken from *Cucumaria miniata* contained numerous crystal cells.

9. *Vibratile cells*

Occasionally small oval cells, ranging from 5 to 7 microns in length and bearing one or two flagella up to 23 microns in length, were observed in fresh preparations of holothurian coelomic fluid. The clear hyaline cytoplasm of these cells was filled with minute yellow granules, 0.5 micron in diameter. No nucleus was observed.

The vibratile cells were not regularly observed in any species, and were never recognized in fixed material.

DISCUSSION

Of the nine types of holothurian coelomocytes described in the literature only five, hemocytes, amoebocytes, morula cells, lymphocytes, and crystal cells, appear to be normal cellular elements in holothurian coelomic fluid. Of these five, two, hemocytes and crystal cells, were found to occur in all holothurians investigated (see Table I). Based on the literature and on the findings of the present investiga-

TABLE I

Coelomocytes found in eleven species of holothurians

Species	Hemocyte	Amoebocyte	Morula cell	Lymphocyte	Crystal cell	Brown body
Order Dendrochirota						
Cucumaria lubrica	×	×	×	×	×	×
Cucumaria miniata	×	×	×	×	×	×
Cucumaria piperata	×	×	×	×	×	×
Eupentacta pseudoquinquesemita	×	×	×	×	×	×
Eupentacta quinquesimita	×	×	×	×	×	×
Pentamera populifera	×	×	×	×	×	×
Psolidium bullatum		×	×	×	×	×
Psolus chitonoides		×	×	×	×	×
Order Aspidochirota						
Parastichopus californicus		×	×	×	×	×
Order Molpadonia						
Molpadia intermedia	×	×	×	×	×	×
Order Apoda						
Leptosynapta clarki		×	×	×		×

tion, hemocytes are apparently limited mainly to certain groups of the Dendrochirota and Molpadonia. Boolootian (1962) gives the first report of hemocytes for a member of order Apoda, *Synapta maculata.* Crystal cells have not been observed in the order Apoda. Because amoebocytes, morula cells and lymphocytes were found in all holothurians in the present investigation, and because of their many reports in the literature, one may conclude that they are likely present in all holothurians. Apparently any coelomocyte type may occur in any of the coelomic compartments in any one organism, although no coelomocytes have yet been observed in the sinuses.

Little doubt exists that minute corpuscles and fusiform bodies are artifacts and, therefore, cannot be considered normal coelomocyte types. Minute corpuscles are formed by the cytolysis of hemocytes and amoebocytes. Fusiform bodies appear to be fragments of torn tissues and show increased numbers in samples of coelomic fluid taken from roughly handled specimens.

Vibratile cells have been reported for *Parastichopus* (*Stichopus*) *californicus* by Kindred (1924), Boolootian and Giese (1958), and Boolootian (1962), and for *Caudina chilensis* by Kawamoto (1927). The irregularity with which such cells were observed in the present investigation leads me to consider them as contaminants or as protozoan symbionts. Observations of flagellated cells with the appearance of vibratile corpuscles increased when care was not taken in rinsing needles and syringes with filtered sea water. Surface contaminants on the holothurian could easily have been introduced into the sample as the needle was inserted in the organism. The possibility exists that some vibratile cells might represent intact or fragmentary cells torn free from the flagellated peritoneum. The majority of the vibratile cells, however, differ markedly from the flagellated peritoneal cells. Most vibratile cells have longer, more conspicuous flagellae than do the peritoneal cells. Vibratile cells also usually contain numerous yellow cytoplasmic inclusions lacking in peritoneal cells.

Brown bodies apparently are masses of degenerating cells laden with yellow-brown granules. They apparently do not represent a separate coelomocyte type, but are formed by the aggregation of degenerating coelomocytes, the most abundant being granule-laden amoebocytes. The yellow granules most likely represent material that is derived from or gathered by amoebocytes, temporarily stored, and eventually eliminated from the holothurian. Brown bodies are probably normally found in the coelomic fluid of all holothurians.

The variety of amoebocytes in holothurians appears to represent different morphological stages of a single coelomocyte type. Petaloid amoebocytes transform directly into the amoebocytes with filiform pseudopodia often listed as a separate category of coelomocytes in the literature. Any classification of coelomocytes that includes a variety of amoebocyte types appears to the author to be artificial. Amoebocytes have been assigned many functions; among these functions are the phagocytosis of foreign materials and tissue debris from the coelomic fluid, the initiation of the clotting process of the coelomic fluid, the absorption or uptake of nutrients from the holothurian digestive tract, and the transport of these nutrients throughout the holothurian body. The author feels that these assigned functions deserve further investigation.

Morula cells are referred to as trephocytes by Liebman (1946, 1947) who assigns them the role of food storage. Lymphocytes apparently have no definitely established function, but seem likely to be the stem cell form from which the other coelomocyte types are derived. Crystal cells have at present no known function nor is the chemical nature of the crystalline material known. The suggestion has been made that some of the cells bearing the more elaborate crystals might be related to the mesenchymal ossicle-forming cells of the body. There is, however, no direct evidence at the present time to support this claim.

The distribution of hemocytes among the holothurians is of interest. Hemocytes are usually assigned the role of oxygen transport, but many actively burrowing forms apparently lack hemocytes as do most of the largest and most active holothurians, the aspidochirotes.

Much remains to be learned concerning the function of the various coelomocyte types before the physiology of holothurians can be fully understood.

SUMMARY

1. The coelomocytes of eleven species of holothurians, representing four orders, were surveyed and described. Eight of these species have not before been surveyed for their coelomocyte contents.

2. The results of the investigation indicate that holothurian coelomocytes fall into five basic categories: hemocytes, amoebocytes, morula calls, lymphocytes, and crystal cells.

3. Brown bodies do not represent a separate coelomocyte type, but are normally found in holothurian coelomic fluid.

4. Amoebocytes, lymphocytes, and morula cells are believed to be present in the coelomic fluid of all holothurians. Hemocytes appear to be limited mainly to certain groups of the orders Dendrochirota and Molpadonia. Crystal cells have not yet been observed in order Apoda.

5. Any coelomocyte type present in a given species may be found in any of the coelomic compartments, with the exception of the hyponeural sinuses in which coelomocytes have not yet been observed.

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THE COMBINED EFFECT OF ULTRAVIOLET IRRADIATION AND HEAT UPON CLEAVAGE OF SEA URCHIN EGGS[1]

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The damaging, even lethal, effect of heat on cells following a sublethal dosage of ultraviolet light was first reported by Bovie and Daland (1923), using very short wave-lengths and 30.5° C. upon *Paramecium caudatum*. Reversing the order of the stimuli had much less effect. They called the effect produced sensitization.[2] Curran and Evans (1938) confirmed Bovie and Daland's finding, employing bacterial spores, heat and wave-length 254 millicrons. Giese and Crossman (1945) later reported the same effect in two species of *Paramecium*.

More recently, Garay and Guba (1951) have sensitized adenosine triphosphate to the splitting action of B-myosin; also, Kleczkowski (1954) has reduced the stability of the chymotrypsin molecule and caused an increased rate of denaturation of tobacco mosaic virus through exposure of these molecules to ultraviolet light and elevated temperature.

The significance of the experiments of this paper lies in the finding of an absence of sensitization of *Arbacia* eggs to a temperature of 36° C. through irradiation with wave-length 254 $m\mu$, and the finding of a tendency toward sensitization in *Strongylocentrotus* eggs when ultraviolet light and a temperature of 31.4° C. were used; also, in an examination of the effects which equivalent doses of heat and ultraviolet light, singly, combined and in alternative order, produce in egg protoplasm.

METHOD

For the *Arbacia* experiments, Westinghouse Sterilamp #WL 782L30, over 91% of whose emission is in wave-lengths 254 $m\mu$, was operated before use for a minimum of 10 minutes to insure constancy of output. Throughout the 9 weeks of experiments the energy liberated was 770 ergs per square millimeter per second, as measured by an ultraviolet light meter which recently had been calibrated. Irradiation, which took place at room temperature, average 24° C., was carried out at a distance of 81 mm. beneath the approximate center of the lamp and in a small circumscribed area. A single layer of eggs, covered by 3 mm. of sea water, was exposed in Syracuse watch glasses. All eggs were concentrated within a 10-mm. radius by rotation of the dishes. The average rise in temperature of the sea water due to heat generated by the ultraviolet lamp was found to be 0.5° C.

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[1] This paper was presented September 1, 1961, as part of the Symposium on Light in the Sea at the Tenth Pacific Science Congress, Honolulu, Hawaii.

[2] Sensitization: The production of an exaggerated injury after sublethal irradiation by a sublethal dose of some secondary agent, and the absence of such exaggerated injury when the agent and irradiation are given in reverse order.

for 120 seconds and 0.6° C. for 200 seconds. The length of exposure to irradiation in different experiments was 40, 80, 120, 160 or 200 seconds.

For the *Strongylocentrotus purpuratus* experiments with the West Coast sea urchin, two small research ultraviolet lamps with 93% of emission at wave-length 254 mμ were used. These were placed 10 cm. above and below the eggs. Eggs were exposed in a small quartz vessel which held, in a single layer, from 1500 to 2000 eggs. By the use of other vessels, two thicknesses of quartz and 2 ml. of sea water were traversed by the ultraviolet light from above and below the eggs. Each 4-cm., nearly circular lamp, constructed of Vycor tubing 1 cm. in diameter, yielded approximately 25 ergs per square millimeter per second when 15 milliamperes of current passed through each lamp tube (wired in series). By means of a Variac in the circuit, voltage could be varied, which permitted delivery of graduated intensities of ultraviolet light within the adopted time interval of ten seconds. The lamps' output was measured by a Hanovia ultraviolet light meter, recently calibrated for 254 mμ radiations. For the standardized 10-second exposure, 250 ergs per square millimeter reached each of the upper and lower surfaces of every egg, approximately 5000 microwatts per square centimeter.

A water-bath of approximately 40 liters capacity, equipped with a blade stirrer, was maintained by means of a thermostat at 36° ± 0.5° C. for *Arbacia*, and at 31.4° ± 0.5° C. for the *Strongylocentrotus* experiments. Thin glass test tubes, calibrated to hold 4 ml., were filled with fresh sea water from 10 to 20 minutes before use and placed in a rack within the water-bath, ample time to permit the tubes of sea water to equilibrate with the temperature of the water-bath.

Sea urchin eggs were inseminated before use and examined for presence of normal fertilization membranes. All *Arbacia* experiments were begun 10 minutes, and *Strongylocentrotus* experiments from 20 to 30 minutes after insemination, since preliminary experiments had indicated that these were especially sensitive periods. All 6 phases of one complete experiment were performed in many cases upon the same batch of eggs. To secure comparable biological effects with heat and ultraviolet light, selection of the graduations in the intensity of each agent was by comparison of one- and two-day-old larvae that had emerged from treated eggs. Size, defects in spines and rate of swimming were the criteria used in establishing comparable biological effects.

Treatments were paired. For instance (a) 10 seconds' irradiation plus exposure to heat for 1, 2, 3, 4 or 5 minutes and the same two agents employed in reverse order constituted one pair of treatments; (b) two minutes' exposure to heat plus exposure to the ultraviolet radiation generated by 5, 15, 25, 35 and 45 milliamperes of current and these same two agents in reverse order constituted a second pair of treatments; (c) the use of each physical agent by itself—heat and ultraviolet light—constituted the third pair of treatments.

The fertilized sea urchin eggs were concentrated by centrifugation. When irradiation preceded heat, the eggs were diluted 75 times; when heat preceded irradiation, the dilution was 38 times. Both dilutions were sufficient to ensure that as each egg, whose diameter is 75 to 80 μ, sank it was heated almost instantaneously to 36.0° C. (*Arbacia*) or to 31.4° C. (*Strongylocentrotus*). The amount of water introduced with 1/10-ml. suspension of eggs was negligible compared with the 4-ml. volume of receiving medium. After the particular temporally-graduated dose of heat was given, contents of the heated tube were poured into a Syracuse watch glass that had been rinsed with sea water and stored at 1° C. for at least one-half hour prior to use. Thus, the heating effect was stopped swiftly since contact with the cold dish brought the temperature of the sea water to 3° C. in 180 seconds. Those eggs which required irradiation were then subjected to the appropriate ultraviolet light treatment. Although the sudden exposure to 1° C. served as a slight stimulus, no analysis of this relatively minor agent has been made since it was a constant factor throughout the experiments.

All controls and experimental dishes containing *Arbacia* eggs were kept in dim light upon a table of running sea water, mean temperature 22° C., whose temperature variation during the course of any one experiment averaged ±0.2° C. *Strongylocentrotus* eggs underwent all treatments in the dark and were reared in darkness at a mean temperature of 12.2° C. ± 0.6° C.

The time at which 50% of the sea urchin eggs underwent the first cleavage was determined by observation of the eggs through a stereoscopic microscope and the count was tallied by the use of hand counters. In fields picked at random the number of divisions observed occurring at one certain time per 100 eggs was counted at least twice (estimated error of 0.5%). In dishes containing more severely damaged eggs, the time at which 50% of first cleavage was attained could be and was plotted graphically. Since it was impossible to decide with accuracy when 50% of the second or third cleavages had been reached by the drastically treated eggs, only data regarding first cleavage were accepted. If an examination of second cleavage of the control eggs revealed the presence of more than 5% of abnormal cleavages, the entire batch of control and experimental eggs was discarded as inferior material.

RESULTS AND INTERPRETATION

In Figure 1 the essential differences in contour of curves between heat (symbol o) and ultraviolet light (symbol ●) are presented. Note that in the controls the average interval between insemination and attainment of first cleavage by 50% of the eggs is 52 minutes in *Arbacia* and 126 minutes in *Strongylocentrotus*. The *Arbacia* heat curve rises slowly at first, followed by a steep change in slope between gradations 3 and 4, which suggests a chemical reaction of the first order. Although no readings between these gradations have been taken, the curve has been drawn in this region with a minimum deviation from the extrapolated extension of each of its limbs. In the ultraviolet curve a sharp rise occurs in the first 10 seconds. Thereafter, the curve ascends slowly and uniformly. The abrupt change in slope seems to indicate an intense initial ultraviolet action upon sea urchin eggs, followed by a more gentle secondary action. The seemingly straight line of the secondary action of ultraviolet light reveals the proportionality between dosage and effect. If one compares the effect of short exposures of eggs to those two physical agents, ultraviolet light causes a greater delay in the onset of cleavage than heat does. Attention is called to the fact that 10 seconds of ultraviolet light caused the delayed first cleavage to occur 73 minutes after insemination of the *Arbacia* eggs and the second gradation of heat caused cleavage to occur 75 minutes after insemination. Thus, each agent which was used as a secondary stimulus produced an equivalent delay in first cleavage.

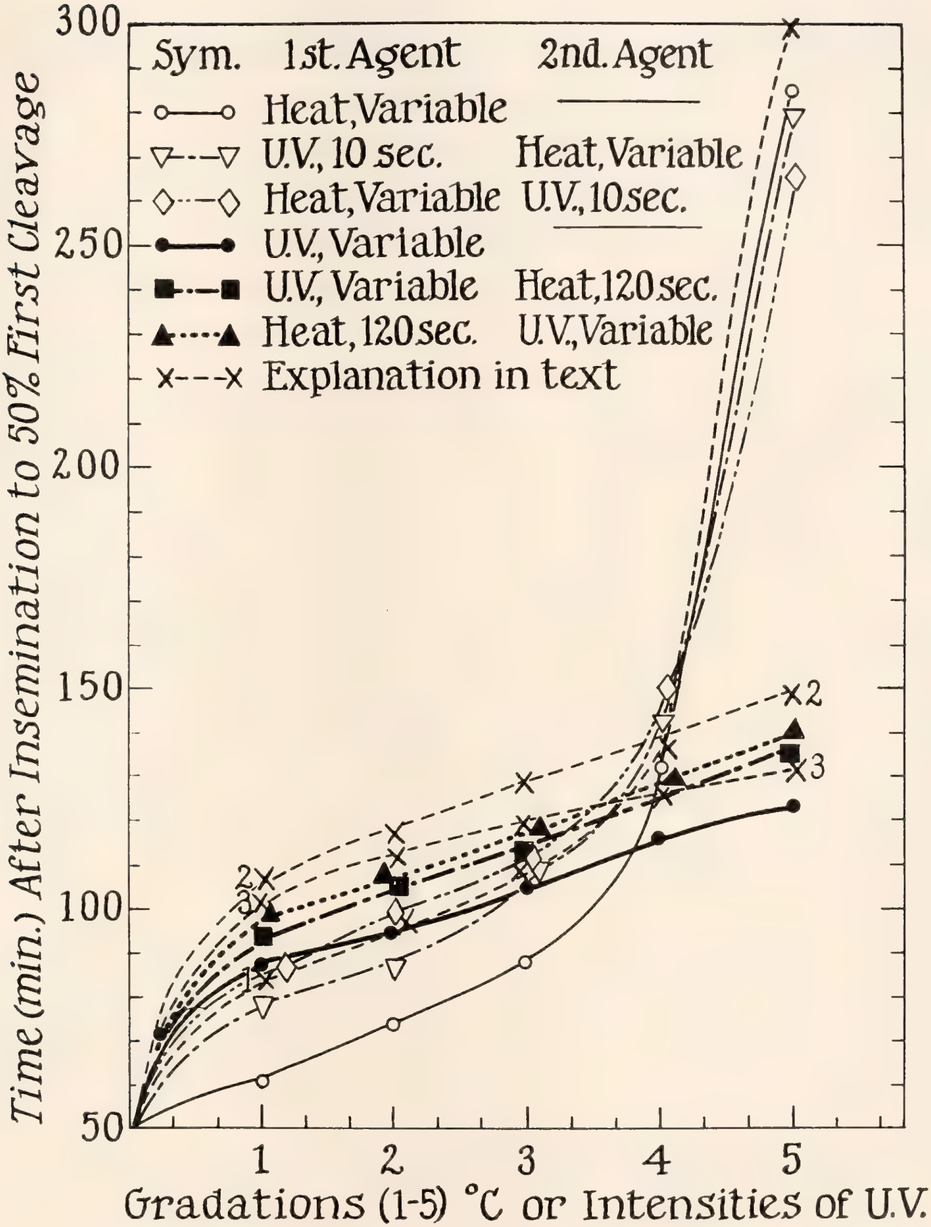

FIGURE 1. *Arbacia* eggs. Basic curves of cleavage delays resulting from exposure to graduated doses of heat or 254 mμ of light. Also, delays caused by combinations of stimuli, applied in the order indicated in the table.

In Figure 1, *Arbacia,* it may readily be observed that the experimental curves represented by solid lines tend to fall into two subfamilies; those in which the heat treatment was given in graded exposures resemble the basic heat curve, and likewise, those in which the ultraviolet treatment was given in graded intensities resemble the basic ultraviolet curve.

Apparently only graduated exposures to ultraviolet irradiation (and heat treatment) produce conditions within a cell which hinder full expression of damage caused by whichever agent is administered secondarily. The 10-second exposure to ultraviolet radiation, which is used as a minor agent in combination with graded exposures of heat, should produce only the initial or intense ultraviolet reaction. Hence, its effect and that of heat should be completely additive. If to the delay produced by heat treatments there is added the 21-minute delay caused by 10 seconds of ultraviolet irradiation, the broken-line curve, numbered 1, is obtained. Since neither curve that represents a combination of graded exposures to heat plus minor ultraviolet radiation differs significantly from the hypothetical curve, numbered 1, the effect of a small dose of ultraviolet light plus exposure to gradations of heat is an additive one. In the curves in which ultraviolet light is given in serial dosages, both initial and successive effects of irradiation are present. Hence, it is reasonable to suppose that if heat and ultraviolet action both were to affect the same cellular constituents, that agent administered after the first one, whether radiant or thermal, would act upon protoplasm already injured by the first agent and could not exert its full retarding effect upon cleavage. Such seems to be the case, for the curves representing graded dosages of ultraviolet light, plus heat as the minor agent, nearly coincide with the broken-line curve numbered 3, and not with the curve numbered 2 which represents a theoretical sum of the delays. The curve numbered 3 represents seven-eighths of the sum of the graded dosages of ultraviolet light and gradation 2 of heat exposure. Experimental results therefore indicate that, except for the briefest of exposures, whenever ultraviolet radiation is employed in graduated doses in combination with a condition of elevated temperature, neither treatment produces its full effect upon the protoplasm of the *Arbacia* egg.

One is forced to conclude that under the conditions of these experiments with *Arbacia* eggs there is absence of sensitization to heat by preliminary irradiation with ultraviolet light of wave-length 254 mμ. Were sensitization present, a heat treatment following irradiation should cause a considerable delay in cleavage over that brought about by the same treatments given in reverse order. But it will be noticed that the two-agent curves of the heat subfamily show no statistically significant differences between them and, likewise, the two-agent curves of the ultraviolet subfamily show only insignificant differences.

However, an examination of the curve representing the effect of treatment of fertilized *Strongylocentrotus* eggs by the fixed 10-second ultraviolet irradiation and graded doses of heat (Fig. 2—symbol ▽) reveals a strong trend toward sensitization. As indicated by the dash-arrow on curve with symbol ▽, no batch of eggs achieved the designated criterion of 50% of first cleavage if pretreated for 10 seconds with ultraviolet light followed by gradations 4 or 5 of heat. Counts of cleavages revealed that in only 33% of the experiments did those eggs which received ultraviolet irradiation before any length of exposure to a temperature

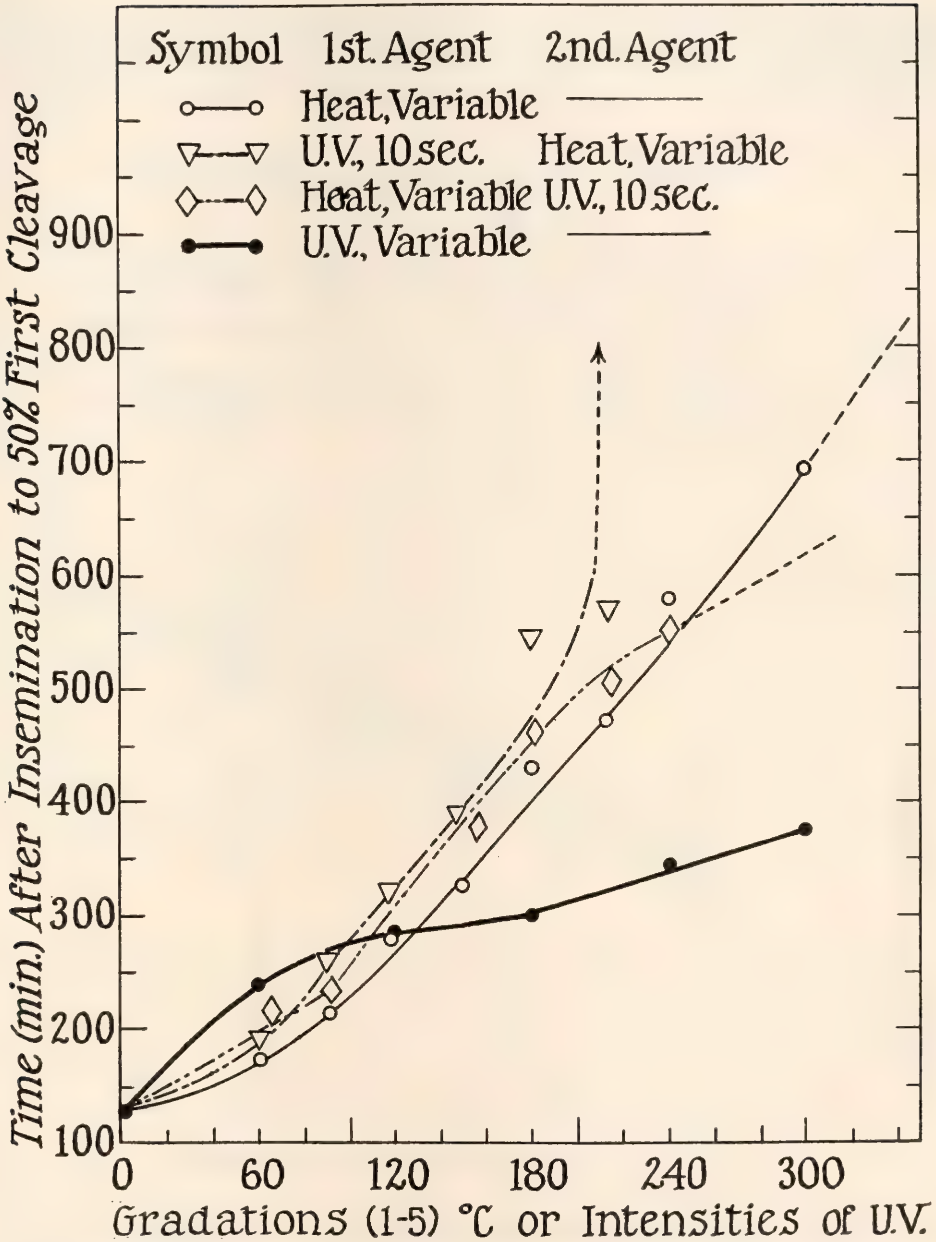

FIGURE 2. *Strongylocentrotus* eggs. Curves representing delays in attaining 50% of first cleavage caused by exposures as indicated in table. Note order of application of stimuli.

of 31.4° ± 0.5° C. reach 50% of first cleavage, whereas among those eggs getting the same dosages but receiving ultraviolet light as the latter treatment nearly all attained the 50% cleavage criterion. Had *Arbacia* eggs been heated at a slightly higher temperature, it is probable that ultraviolet light sensitization to heat would

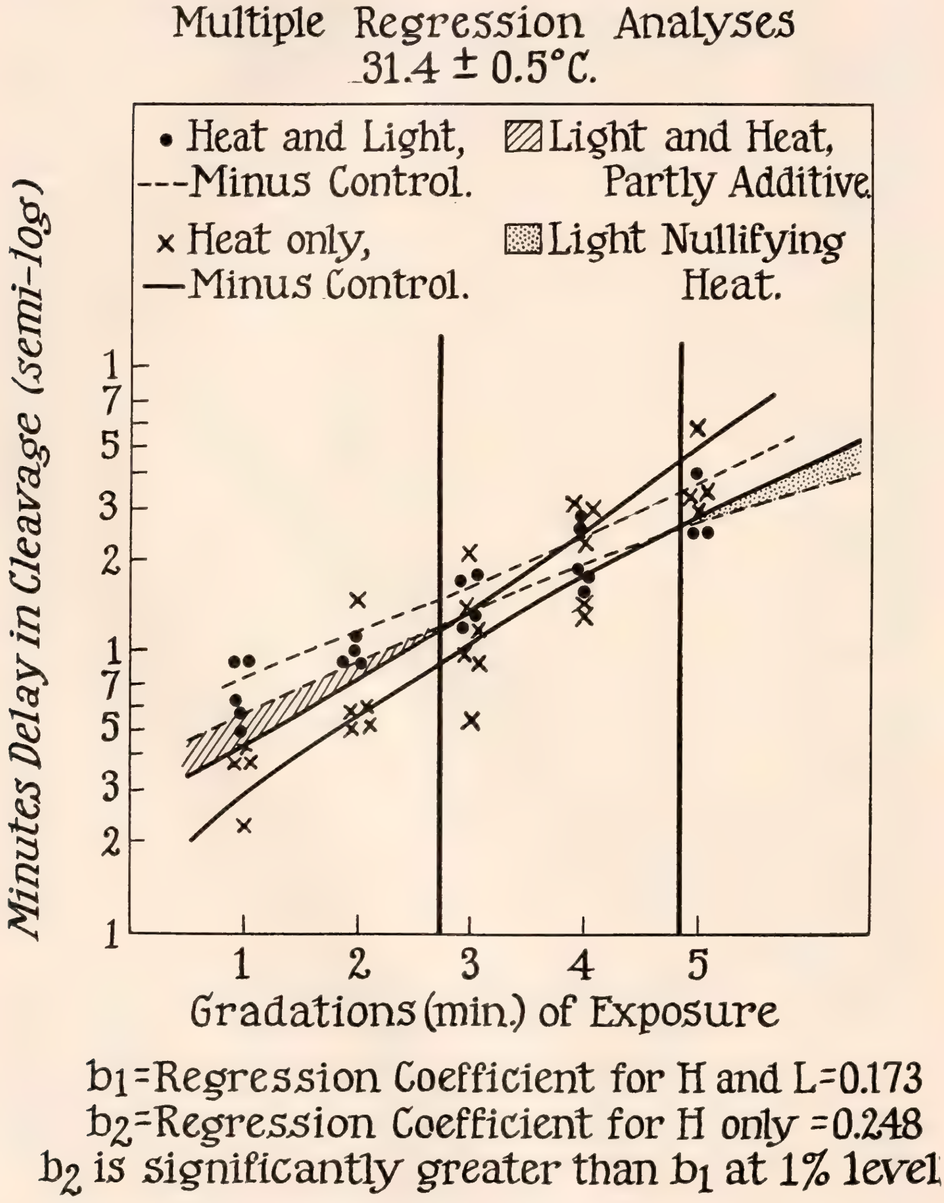

FIGURE 3. Multiple regression analyses of *Strongylocentrotus* data regarding delays in attaining 50% of first cleavage.

have been shown, similar to that revealed by a multiple regression analysis of the above-mentioned *Strongylocentrotus* data. In view of the fact that light treatment alone should produce from 50 to 80 minutes delay in cleavage, it seems likely that the heat-plus-light treatment delayed cleavage at least an hour *less* than would be expected if the heat and light effects were independent and linearly additive. Attention is called, also, to the crossing of the heat-plus-ultraviolet light curve (Fig. 2—symbol ◇) below the heat-only curve at gradation 3½ (230 seconds), a difference which is indicative but not statistically significant.

A semilogarithmic plot of the heat curve of both *Arbacia* and *Strongylocentrotus* yielded two straight lines; only gradation 5 of *Arbacia* did not lie on its exponential curve. The slopes for these lines—*Strongylocentrotus,* 0.166 and *Arbacia,* 0.102—revealed that *Strongylocentrotus* eggs, living customarily at a lower temperature than *Arbacia* eggs, were damaged more than the latter by heat administered in graduated dosages. *Arbacia*'s first cleavage at 52 minutes in contrast to *Strongylocentrotus*' at 126 minutes showed the response to temperature that would be expected of animals reared at 22° C. and 12.5° C., respectively. For this temperature separation of about 10 degrees, the predicted Q_{10} of approximately 2 occurred.

Figure 3 indicates that the regression coefficient for heat-only is significantly greater at the 1% level than the regression coefficient for heat followed by 10 seconds of ultraviolet light. Since *Strongylocentrotus* eggs which have been subjected to both heat (31.4° ± 0.5° C.) and light treatments show significantly less additional delay in achieving 50% of first cleavage as the time of exposure is increased than do those which have undergone only heat exposures, this indicates that the addition of light diminishes the harmful effects of heat when the whole range from 1 to 5 minutes is considered.

That recovery from mild injuries caused by the two physical agents, used singly or combined, does occur could be observed in the large percentage of normal or nearly normal sea urchin embryos and plutei obtained. Survivors from among the more drastically treated eggs revealed evidence of exposure to heat and ultraviolet light by their small size, shortened or missing spines and other blemishes.

Discussion

That the effects of ultraviolet irradiation are multiple (Heinmets and Nathan, 1954) has been brought out clearly by data published in the last decade. Cytolysis, mentioned by Blum, Cook and Loos (1954), the clumping of pigment of the cortex, reabsorption of intercellular boundaries, as well as delays in cleavage and abnormal cleavage patterns, were evident in the experimental material of this paper.

Probably, the most important effect of ultraviolet irradiation is upon nucleic acids. Iverson (1957), using *Tetrahymena pyriformis,* found that DNA synthesis was inhibited by doses of wave-length 254 mμ that permitted a continuation of cellular enlargement, also, that RNA synthesis ceased, following irradiation, after about one division cycle. Since DNA functions in the replication of chromosomes, cleavage could not occur until sea urchin eggs had recovered from insult to nucleotide precursors of DNA.

Novick and Szilard (1949) have postulated the formation in protoplasm of both a stable and a labile poison by treatment with ultraviolet light. The labile poison was considered to be rendered non-toxic by white light. Since it has been shown that post-treatment of *Strongylocentrotus* eggs with ultraviolet light of the germicidal wave-length, 254 mμ, significantly reduces a delay in attaining first cleavage that was caused by heat, the formation of a stable poison through the action of ultraviolet light seems questionable.

The writer thanks the trustees of the Marine Biological Laboratory, Woods Hole, Massachusetts, and the Friday Harbor Laboratories of the University of Washington for the use of their facilities; also, the Cleveland Area Heart Society for its grant.

SUMMARY

1. Thermal treatment (36° C. *Arbacia;* 31.4° C. *Strongylocentrotus*) applied to sea urchin eggs in graduated exposures causes a delay in first cleavage that is roughly proportional to the exposure.

2. Graduated intensities of 254 mμ of ultraviolet light produce a delay in first cleavage that is less pronounced with increasing intensities of light.

3. Treatments involving both ultraviolet irradiation and heat retard the first cleavage of sea urchin eggs more than either agent alone.

4. Curves of cleavage delays of *Arbacia* eggs caused by combined ultraviolet light and 36° C., either one serving as the major physical agent and the second as the minor physical agent, show no statistically significant difference from a hypothetical curve representing a sum of the two single-agent delays.

5. Although there is no evidence that ultraviolet irradiation preceding heat causes an exaggerated injury, which application of the same agents in reverse order fails to do with *Arbacia* eggs, there is an indication that such sensitization tends to occur in *Strongylocentrotus* eggs pretreated with 254 mμ of ultraviolet radiation and then subjected to heat at 31.4° ± 0.5° C.

6. *Strongylocentrotus* eggs pretreated to a temperature of 31.4° C., followed by exposure to ultraviolet irradiation (254 mμ), show significantly *less* delay in achieving first cleavage than eggs subjected only to a temperature of 31.4° C.

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SOUND PRODUCTION IN THE SNAPPING SHRIMPS ALPHEUS (CRANGON) AND SYNALPHEUS [1,2]

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Many marine organisms are sources of sound in the sea, especially fishes and mammals. Among the invertebrates, the Crustacea include the most prodigious sound producers, for example, the spiny lobsters (Palinuridae) (Dijkgraaf, 1955; Moulton, 1957; Hazlett and Winn, 1962), stomatopods, and some crabs. But the sounds of these are not so persistent and widespread as the noise produced by snapping or pistol shrimps of the genera *Alpheus* (*Crangon*) and *Synalpheus,* which inhabit warmer seas.

Although it has long been known that a snapping shrimp is capable of emitting a loud "snap", the "crackling" noise produced by vast numbers of these shrimps was formerly attributed to a variety of things, *e.g.,* water noise, *Teredo* boring on ship hulls, terrestrial disturbances, and many other phenomena. Hulburt (1943) speaks of a crackling noise similar to that produced by "dragging a blackberry bush" which he heard at various places along the southern coast of the United States.

Early in 1942 a similar crackling noise of unknown origin was heard in various localities off the coast of California. F. A. Everest, R. H. Fleming, M. W. Johnson, and R. W. Young, of the University of California's Division of War Research, determined that the sound was due to a continuous fusillade of snaps from millions of snapping shimps (Johnson, 1943; Everest, Young and Johnson, 1948).

Snapping shrimp sounds have been analyzed by several investigators (Loye and Proudfoot, 1946; Johnson *et al.,* 1947; Everest *et al.,* 1948; Johnson, 1948; Knudsen *et al.,* 1948; Dobrin, 1949; Shishkova, 1958). The present paper deals primarily with the characteristics of snapping shrimp sounds of Bermuda waters, and examines further the derivation of ecological data from the analysis of underwater sounds. The anatomy and physiology of the sound-producing organ are also discussed.

The sounds analyzed in this study were recorded at Bermuda from June to August, 1958. The recording equipment consisted of an AX-58-C Rochelle salt hydrophone, a Woods Hole suitcase amplifier, and an Ekotape tape recorder model 205 or a PT6-BN and a PT6BA2HZ Magnecorder tape recorder. Recordings were made so that the nature of the sounds could be determined for different

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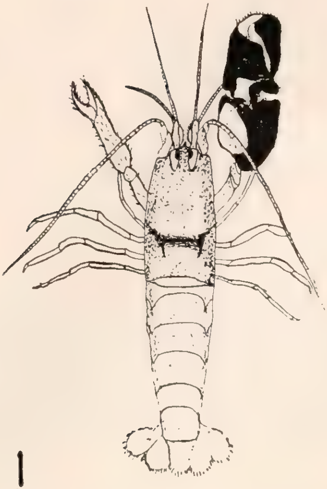
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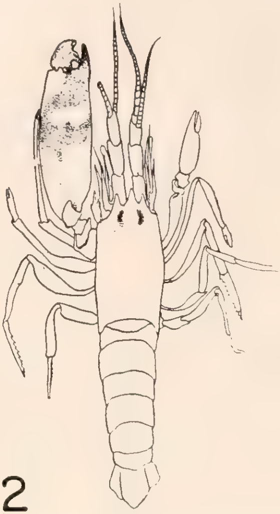
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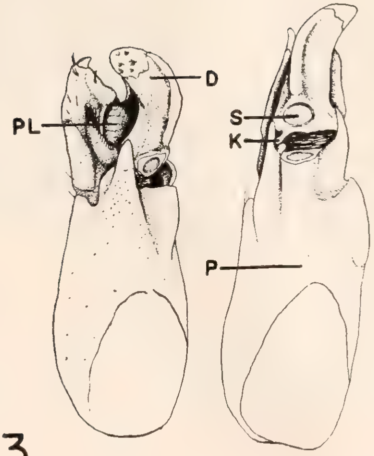

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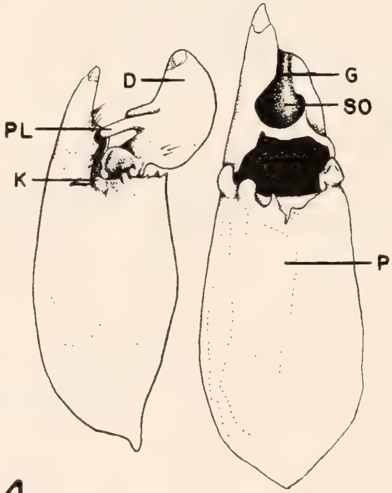

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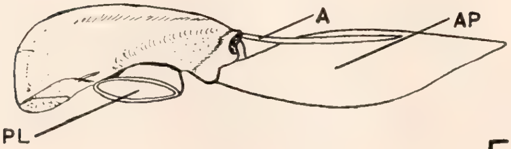

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Figures 1–5.

depths and bottom conditions. Single snaps recorded in the laboratory were also studied. The recordings obtained were analyzed on a Kay Vibralyzer vibration frequency analyzer.

STRUCTUURE OF THE SOUND-PRODUCING CHELA

The first pereiopods of the members of the Family Alpheidae, to which the snapping shrimps belong, bear dissimilarly enlarged chelae. The larger claw, a weapon of offense and defense, produces the snapping sound. Its anatomy, from the point of view of sound production, has been partially elucidated (Volz, 1938; Johnson *et al.*, 1947; Hazlett and Winn, 1962). MacGinitie (1937) observed this claw's use as a weapon of food capture (see MacGinitie and MacGinitie, 1949, pp. 276–279, for further discussion of the animal's habits.) The smaller claw is used mainly in grasping and handling objects. In *Alpheus heterochelis* and *A. normanni,* of North Carolina, approximately half of a chance collection will be "right-handed" (the claw on the right will be the larger one in a dorsal view, as in Figure 1) and the other half will be "left-handed." It has been shown for several species of *Alpheus* and *Synalpheus* that when the large chela or crusher is removed, a small chela or nipper is regenerated from the stump of the larger one, the original nipper differentiating into a crusher at the next molt. This "chela reversal" was first noted by Przibram (1901), and several hypotheses have been advanced in explanation (Wilson, 1903; Darby, 1934; Dawes, 1934). Chela reversal is doubtless of biological advantage to the animal, for it permits more rapid formation of the crushing claw than would otherwise be the case.

It is of interest to note that the dactylopodite or "movable finger" of the *Alpheus* and *Synalpheus* snapping chela lies laterally on the propodite or "hand," in contrast to other typical decapods, in which it lies medially. This difference is due to a very flexible joint between the propodus and carpus. In many decapods this joint is hinge-like, and the dactylus is always held medially. In alpheids, on the other hand, flexibility of the joint allows the animal to rotate the propodite 180°, so that the dactylopodite can be held either laterad or mediad to the body. Although the dactylopodite is normally held laterad, turning over the propodite so that the dactylopodite is mediad causes no apparent injury or annoyance to the animal; however, we have not observed a shrimp snapping its chela in the inverted position.

The portions of the snapping chela that are most important in production of sound are the propodus and the dactylus. In both *Alpheus* and *Synalpheus* these portions of the large chela are proportionately enormous, but there are distinct anatomical differences between the two genera which have not hitherto been pointed

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FIGURE 1. *Alpheus heterochelis,* dorsal view, × 2. After Brooks (Brooks and Herrick, 1891).

FIGURE 2. *Synalpheus minus,* dorsal view, × 5. Drawn from life.

FIGURE 3. Large chela of *Alpheus peasei,* lateral (left) and dorsal (right) views, × 6. Drawn from life. D, dactylus; P, propodus; PL, plunger; S, oval disc; K, exoskeletal knob.

FIGURE 4. Large chela of *Synalpheus minus,* lateral (left) and dorsal (right) views, lateral view with dactylus removed to show water jet groove, × 15. Drawn from life. D, dactylus; P, propodus; PL, plunger; SO, socket; K, exoskeletal knob; G, water jet groove.

FIGURE 5. Dactylus of large chela of *Alpheus californiensis* showing exoskeletal extensions, dorsal view, × 8. Drawn from life. PL, plunger; A, apodeme; AP, apodemal plate.

out and which are reflected in respective acoustical spectra. The chela is generally ellipsoidal and more swollen in *Synalpheus* (Fig. 2) than in *Alpheus,* where it is somewhat compressed (Fig. 1). *Synalpheus* chelae are simple and have no notches or grooves, but those of *Alpheus* species are usually notched and deeply grooved (Fig. 3).

Another generic difference lies in the arrangement of the tips of the terminal articles. In species of *Synalpheus,* the chela tips are directly opposed, but in species of *Alpheus* we have studied, the closing surfaces are arranged somewhat like shears. The distal part of the dactylus closes on a chitinous projection from the propodus. Consequently, in *Alpheus* the sides of the chela tips come together.

Both *Alpheus* and *Synalpheus* possess a tooth-like "plunger" on the dactylus which fits into a "socket" or pit on the propodus when closed. The structure of the plunger and socket differs in the two genera. In *Synalpheus* the plunger is subdivided into a double rod (Fig. 4), while in *Alpheus* it is oval and single (Fig. 3). The socket in *Synalpheus* is narrow to accommodate the thin rod, but in *Alpheus* it is larger and deeper since the plunger is bigger and longer. There are other minor differences which reflect a greater degree of specialization in *Alpheus*.

The Snapping Mechanism

Even at present the exact mechanism by which the snap is produced is uncertain. An early hypothesis attributed the sound to a rubbing of the basal spines of the uropods (Lovett, 1886), and Miner (1950, p. 494) erroneously states that it is caused by a flicking of the wrist joint of the crusher claw. In the living animal, one can easily see that movement of the dactylus of the large claw immediately precedes the snap.

The first careful observations on the way in which the alpheid chela produces sound were made by Kent (1877), but he erroneously attributed it to a sudden opening of the dactylus. Herrick (Brooks and Herrick, 1891) was perhaps the first to correctly describe how the sound is produced. As he points out, the sound is caused by impact of the hardened claw tips coming together as the plunger of the dactylus is driven into the socket of the propodus. Anterior to the socket is a groove through which a jet of water escapes when the plunger suddenly clamps down on the socket (Fig. 4). The main function of the plunger-socket mechanism is not production of sound, but of this water jet, which stuns or frightens enemies. The sound which is produced simultaneously is a "by-product" of this protective movement (Schmitt, 1931, p. 192; Johnson *et al.,* 1947; Moulton, 1957).

Closure of the large chela works much like the uncocking of a pistol hammer. Mechanics of the cocking mechanism, however, are still in doubt. Courtière (1899), Verrill (1922), and Johnson *et al.* (1947) maintain that two oval discs or hardened surfaces (Fig. 3), which come in contact with each other when the dactylus is raised to its fullest extent, serve as "suckers." According to this view, when the dactylopodite is raised, these surfaces adhere to each other like two panes of damp glass. Extra tension must be built up by the adductor muscle in order to break the contact between the suckers, and this increases the impact force. These hardened discs probably do not serve this function, however. They are absent in species of *Synalpheus,* which are also capable of vigorous snapping, and they are present in *Amphibetaeus* species, which are incapable of snapping. The suggestion by Johnson *et al.* (1947) that analogous smooth surfaces of *Synalpheus* serve the same purpose as the "suckers" of *Alpheus* we cannot verify, nor could we demonstrate any suction mechanism in preserved specimens of *A. californiensis,* which were studied by them, and *A. heterochelis.* We found that the dactylus could be moved freely in and out of the well, a fact observed also by Herrick (Brooks and Herrick, 1891). Further, it would be expected that if these discs were functional in producing suction, they would be membranous in character; they seem to be chitinous and no different from the rest of the exoskeleton. It is possible that in the living animal, when the dactylopodite is pulled up and comes in contact with the propodite, the membrane connecting the segments depresses. This depression may cause a partial suction to build up and hold the dactylus and propodus together until the muscular tension is sufficient to overcome the additional resistance.

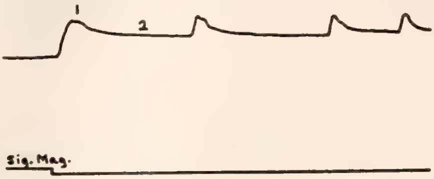
In *Synalpheus* a type of cocking device depending on overlap of exoskeletal knobs may serve to hold the dactylopodite in place. In two species of *Synalpheus* (*S. minus* and *S. goodei*) there are white exoskeletal projections lying dorsad on the chela (Fig. 4). Matched protuberances on the propodus and dactylus slide over each other easily as the dactylus is opened, and they serve to hold it in the raised position against considerable tension As contraction overcomes friction thus provided by the cocking device, the dactylus closes and a snap results. These disarticulating knobs are not equally evident in all species of *Alpheus,* although a similar mechanism may occur throughout the genus.

In addition to the investigators mentioned above, Dobrin (1949) and Moulton (1959) studied the mechanism producing the noise, but knowledge regarding the exact nature of the snapping mechanism still remains scanty, mostly because the many speculations have not been proved by experimentation.

Anatomy and Physiology of the Chela Musculature

Both the abductor and adductor muscle fibers of the dactylus originate on the dorsal and ventral walls of the propodus. The abductor fibers have their insertions on an exoskeletal extension of the dactylus called the apodeme, which is attached to the outer basal angle of the dactylopodite. The muscle fibers of the adductor are more numerous and are inserted on both sides of a large, flat apodemal plate attached to the inner basal angle of the dactylus (Fig. 5).

Physiological studies on the large chela of *A. heterochelis* indicate that the characteristics and innervation of the claw muscles are probably no different from other Crustacea (see Prosser, 1950, pp. 595–614, for a discussion of crustacean muscle and innervation). Both fast and slow types of contraction were demonstrated when the adductor muscle of an isolated *A. heterochelis* claw was stimulated with electrical shocks from an electronic stimulator (Fig. 7). The fast twitch takes place in about 0.1 second, while the slow contraction lasts for more than a second and may be even longer, depending upon duration of the stimulus. This muscle is thus doubly excitable, *i.e.,* it exhibits two distinct types of response, depending upon intensity and frequency of the stimulus. The dactylus adductor is equivalent to two physiologically differentiated units. Separation into slow and fast movements has decided functional significance. The slow system is used in normal movements of the dactylus. The fast system is brought into play when great tensions are needed to crush objects. That the muscle is innervated by separate fast and slow fibers (as in many other crustaceans) has not been demonstrated histologically. Indeed, a bifunctional single nerve fiber system may be present in snapping shrimp chela musculature. However, the double excitable system is more efficient and it exists in crustacean muscles which develop considerable power. In order to create rapid movements, it seems logical that a special fast fiber system would be advantageous in the muscle.



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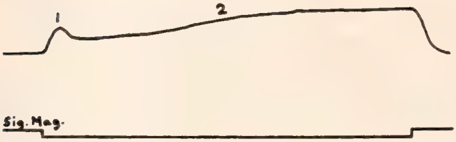

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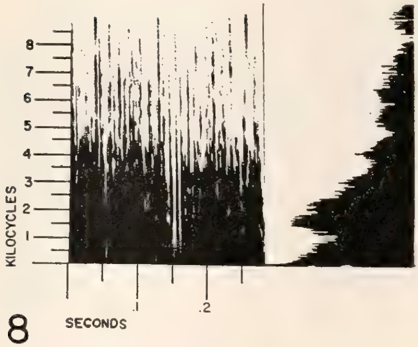
FIGURE 6. Tracing of kymograph record showing fast and slow contractions in adductor muscle of dactylus of *Alpheus heterochelis* large chela.

FIGURE 7. Tracing of kymograph record showing inhibitory effect in adductor muscle of dactylus of *Alpheus heterochelis* large chela.

When the adductor muscle of *A. heterochelis* was stimulated, fast contractions occasionally appeared superimposed on the slow contraction. This effect can be seen in Figure 6. It may be due to simultaneous excitations of both excitatory and inhibitory fibers. The initial contraction (represented by 1) is of the fast type. The tension developed is maintained by the slow contraction (represented by 2). But in this case there is very little summation in the slow contraction, unlike that shown in Figure 7. This may be due to inhibition of the slow contraction by stimulation of inhibitory fibers. The inhibitory effect is overcome when another fast contraction results from stimulation of an excitatory fiber, as shown by the next fast contraction. Possibly the re-emergence of the fast contraction may be due to an elimination of an inhibitory substance by an excitatory substance and the subsequent accumulation of the excitatory substance. A similar rhythmic response was recorded by F. P. Knowlton (1942) on crab claw muscle. These rhythmic contractions are believed to have been due to simultaneous stimulation of inhibitory fibers or nerve endings (F. P. Knowlton and Campbell, 1929).



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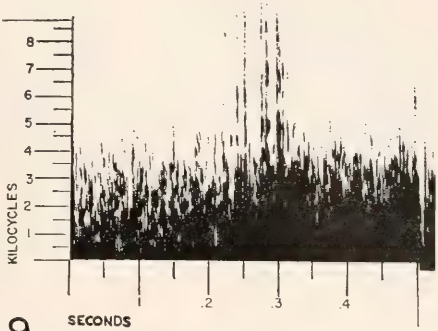

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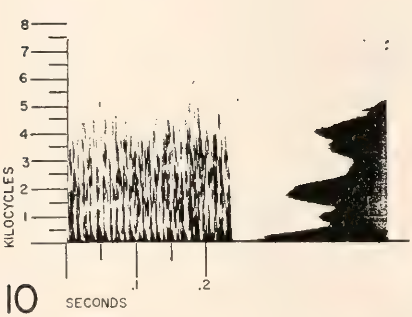

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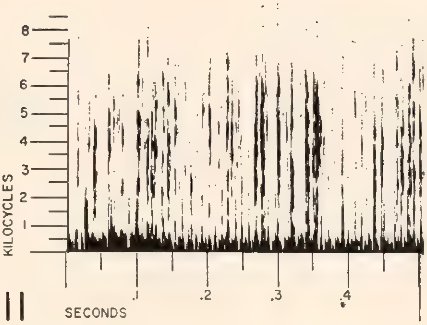

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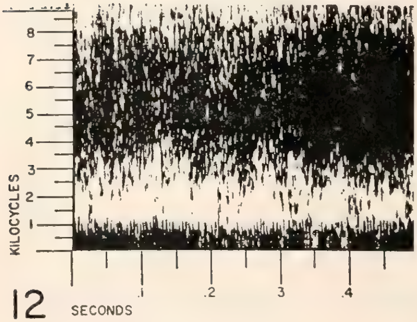

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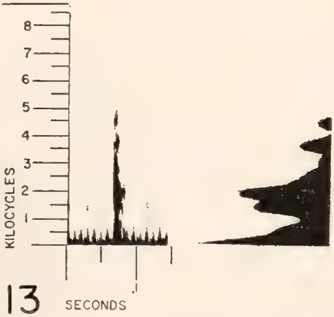

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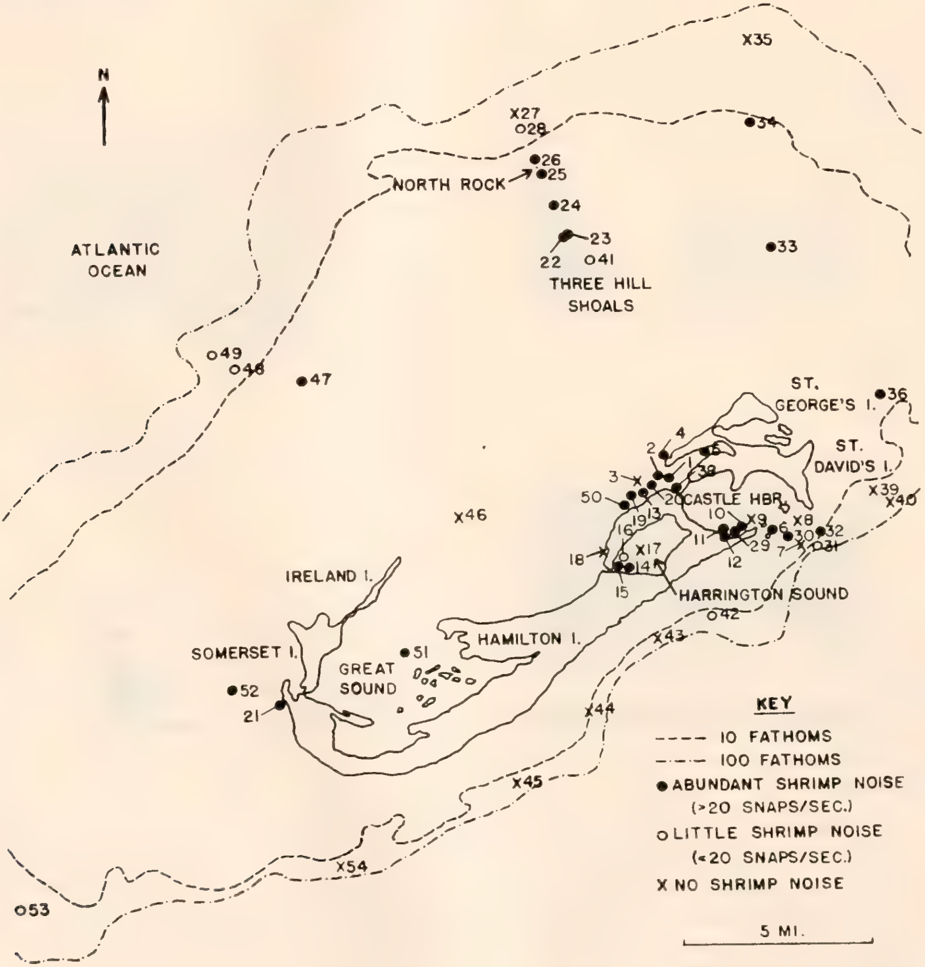
FIGURE 8. Vibragram (left) and section (right) of snapping shrimp noise.
FIGURE 9. Vibragram showing snapping shrimp "volleys."
FIGURE 10. Vibragram and section of water noise.
FIGURE 11. Vibragram of snapping shrimp noise with water noise at a minimum.
FIGURE 12. Vibragram of snapping shrip noise at Bimini, Bahama Islands.
FIGURE 13. Vibragram and section of a single *Synalpheus minus* snap.

Recording and Analysis of Bermuda Snapping Shrimp Noise

Recordings were made at 56 stations surrounding the Bermuda, Challenger, and Argus (Plantagenant) Banks in the Western Atlantic Ocean. During the recordings the hydrophone just cleared the bottom. Sound waves picked up by the hydrophone were recorded on the tape recorder either at 7½ or 3¾ inches per second. Snapping shrimp noise did not occur at all stations, but when it was present it remained fairly continuous throughout the recording. Other noises heard in conjunction with snapping shrimp noise include *Holocentrus ascensionis* (squirrelfish) volleys, *Epinephalus striatus* (Nassau grouper) grunts, *Panulirus argus* (spiny lobster) stridulations, and *Gonodactylus oerstedii* (mantis shrimp) raptorial sounds.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IRELAND I.
HARRINGTON SOUND
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X43
SOMERSET I.
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HAMILTON I.
GREAT SOUND
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KEY
---- 10 FATHOMS
-·-·- 100 FATHOMS
● ABUNDANT SHRIMP NOISE (>20 SNAPS/SEC.)
O LITTLE SHRIMP NOISE (<20 SNAPS/SEC.)
X NO SHRIMP NOISE
5 MI.

Figure 14. Map of Bermuda showing location of the 56 stations and the amount of noise heard at each station. Station 37 (Ariadne Bank), located to northeast, stations 55 and 56 (Challenger and Argus Banks), located to southwest off the map.

Figure 8 represents a typical spectrum of snapping shrimp noise, as analyzed by the Vibralyzer. To the left is a frequency-time portrayal known as a vibragram, where time is plotted on the horizontal axis and frequency on the vertical axis. Each vertical "spike" (which is seen most clearly in the upper half of the vibragram) represents a snap from an individual snapping shrimp. To the right of the vibragram in Figure 8 is a section which reveals the relationship between intensity and frequency at a preselected point in time. A qualitative measure of relative intensity is portrayed on the horizontal axis in a linear scale and frequency on the vertical axis.

A random 1.2-second interval of shrimp noise from each station was recorded onto and analyzed by the Vibralyzer. The frequency range of every vibragram and section made was 88–8800 cycles per second. In all vibragrams band-width is wide, shape flat, and pattern normal. One vibragram and sections of two or three points in the vibragram were made at each station.

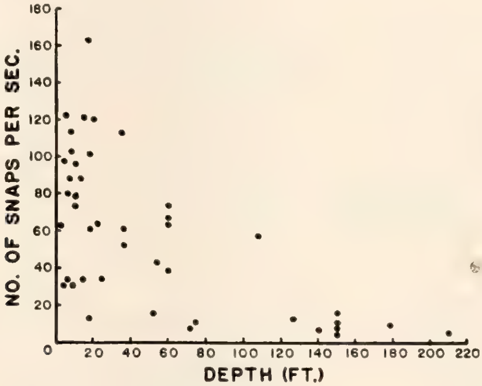


Figure 15. Distribution of shrimp noise with depth.

As a relative measure of the number of snapping shrimp at a particular station, the "spikes" in each vibragram were counted. The total number counted was divided by 1.2 to obtain the average number of snaps per second at each station. They were counted at about the 7 kc level, where the components do not fuse together or mix with water noise, as in the lower frequencies (lower half of Figure 8). One section from each station was analyzed using a grid to show relationship between frequency and intensity. Sections were also taken at stations where no shrimp noise was heard. Relative intensity was measured at 1-kc intervals. Values obtained at each kc were averaged together for stations where snapping shrimp were heard to obtain an average spectrum of snapping shrimp noise. A similar procedure was followed for the 17 stations where noise was entirely or nearly all water noise. Frequencies at which shrimp noise and water noise were most intense were also noted for each station and averaged together.

RESULTS OF THE VIBRAGRAM AND SECTION ANALYSIS

A description of the localities at which recordings were made and the number of snaps per second counted, as well as the point of highest intensity, are given

TABLE I

A description of the localities of snapping shrimp recordings at Bermuda during the summer of 1958, the number of snaps/second counted, and the frequencies of highest intensity, as well as relative intensity

Station number	Location	Depth (ft.)	Bottom conditions	No. of snaps per sec.	Pt. of highest intensity	
					Freq. (kc)	Rel. int.
1	Inside western entrance to Ferry Reach	8	Sandy, near submerged rock and sponge clump	103	3.0	4.5
2	Outside western entrance to Ferry Reach	20	Rocky	120	3.2	3.5
3	Directly in front of Ferry Reach	48	Level sand, some grass and low coral	—	4.3	2.7
4	Whalebone Bay	7	Large coral reef	88	3.2	4.3
5	Dock of Bermuda Biological Station	15	Wharf made from rock piling	121	4.5	3.8
6	Southeast of Castle Island	17	Rocky, some sand and grass	163	4.8	2.7
7	Southwest of Castle Roads	60	Sand, some rocks	—	1.8	3.8
8	Northeast of Gurnet Rock	20	Rocky	—	3.1	1.3
9	Castle Harbor over Great Shoal	2	Shoal	—	0.5	5.1
10	Mouth of Wallace Bay	6	Rock ledges	80	1.9	6.0
11	Little Harbor in Tuckerstown	25	Rock ledges	35	2.2	2.9
12	Mouth of narrow reach north of opening to Tuckerstown Bay	4	Rock ledges	31	3.5	2.1
13	Bailey's Bay between Bay Island and East Point	4	Rock and sand	97	3.5	3.8
14	Harrington Sound side of Flatts Bridge	35	Rocky with bottom growth	113	2.7	4.7
15	West of Flatts Inlet	5	Rock ledges	122	3.5	2.1
16	Green Bay of Harrington Sound	18	Rock ledges and deep cave	12	2.2	3.5
17	Harrington Sound straight out from Green Bay	55	Sand	—	1.0	2.9
18	East side of Shelly Bay	10	Shoal	—	0.8	5.0
19	East side of Bay Island	13	Rocky	88	2.1	4.7
20	West side of Bay Island	2	Rocky	63	2.0	5.3
21	South of Wreck Hill	8	Shallow coral reef	113	0.8	5.5
22	North reefs short of North Rock (recorded in early evening)	10	Shallow coral reef	96	1.7	5.6
23	North reefs short of North Rock (recorded during daytime)	10	Shallow coral reef	73	1.3	5.8
24	North reefs short of North Rock	18	Broken bottom	101	1.4	4.0
25	South of North Rock	10	Broken bottom	78	2.7	5.8
26	North of North Rock	18	Rock ledges	61	1.9	5.6
27	Northwest of North Rock	360	Edge of Bermuda Bank	—	0.0	5.3
28a	Northwest of North Rock	168	Edge of Bermuda Bank	9	1.9	5.4
28b	Northwest of North Rock	126	Edge of Bermuda Bank	12	0.2	6.2
28c	Northwest of North Rock	108	Edge of Bermuda Bank	57	1.7	4.9
28d	Northwest of North Rock	210	Edge of Bermuda Bank	4	1.9	4.9

TABLE I—(*Continued*)

Station number	Location	Depth (ft.)	Bottom conditions	No. of snaps per sec.	Pt. of highest intensity	
					Freq. (kc)	Rel. int.
28e	Northwest of North Rock	150	Edge of Bermuda Bank	15	0.8	4.0
29	Mouth of Wallace Bay	22	Just off rock ledges over sand	64	1.7	5.8
30	Castle Roads south of Charles Island and southwest of Gurnet Rock	54	Sand and sponges	43	1.9	5.2
31	Castle Roads southeast of Charles Island	150	Sand and scattered bottom growth, some rocks	7	0.4	5.5
32	Castle Roads southeast of Castle Island and south of Nonesuch Island	60	Rocky coral broken by level patches of sand	38	3.5	2.9
33	North of St. Catherine's Point	36	Coral reef	52	2.9	5.6
34	North of St. Catherine's Point	60	Rocky	67	3.0	4.8
35	North of St. Catherine's Point	150	Rock ledges	—	0.0	3.9
36	Northeast of St. George's Harbor	60	Rocky	63	1.0	5.7
37	Northeast of St. George's Harbor near Ariadne Bank	140	Rocky	6	1.8	5.2
38	Ferry Reach	9	Old anchored barge	30	4.3	3.2
39	Southwest of St. David's Island	70	Sand, scattered rocks	—	1.9	4.2
40	Southwest of St. David's Island	138	Sand, scattered rocks	—	2.4	3.1
41	North Reefs near Three Hill Shoals	54	Sunken wreck	15	3.8	4.9
42	South of Sam Hall's Bay	72	Rock ledges and sand	7	1.7	4.4
43	South of Town Hill	144	Sand	—	1.9	3.2
44	Southeast of Elbow Beach	60	Sand	—	2.3	2.6
45	White Cliffs toward Warwick Long Bay	138	Sand	—	0.5	4.7
46	Brackish Pond Flats vicinity in northwest harbor basin	45	Sand	—	2.0	2.8
47	Inside northwest reefs	6	Rock ledges	34	2.3	5.4
48	Outside northwest reefs	75	Sand and some rocks	11	0.9	5.2
49	Outside northwest reefs	150	Rock ledges	10	1.8	5.3
50	Bailey's Bay	15	Sand and some rocks	34	2.3	3.0
51	North of Pearl Island	36	Coral reef	61	1.5	4.8
52	West of Wreck Hill	60	Coral reef	73	1.7	4.5
53	Southeast of Wreck Hill	150	Rock ledges	3	0.7	5.8
54	South of Wreck Hill	84	Rock ledges and sand	—	0.8	5.2
55	Challenger Bank	168	Coral reef	—	0.8	3.6
56	Argus Bank	168	Coral reef	—	1.0	4.0

in Table I. Location of each of the 56 stations as well as the amount of noise heard at each station is shown in Figure 14.

DEPTH

Figure 15, in which relative abundance of snapping shrimp (measured by the number of snaps per second) at each of 46 places where shrimp noise was heard is plotted against the depth of each station, indicates depths most favorable and least favorable to snapping shrimp populations. Above 120 feet there is a great deal of variation in the abundance of snapping shrimps. Except for six stations at less than 120 feet, shrimp crackle was very intense. At some of these stations it could be heard above the surface of the water by ear. The noise was most intense in Ferry Reach, near Castle Island, and near Flatts Bridge. At all stations where depth exceeded 120 feet, there was little shrimp noise (less than 20 snaps per second) or none at all.

Data from trawling and dredging collections indicate that most of the species occur in the 0–30 fathom range. Acoustical data corroborate this finding. Loye and Proudfoot (1946), Johnson *et al.* (1947), Everest *et al.* (1948), and Knudsen *et al.* (1948) have shown that sound levels are low in water deeper than 180 feet, even if bottom conditions are favorable. Knudsen *et al.* found that "highest noise levels appear to occur in water between 30 and 140 feet deep." However, in Bermuda, shrimp crackle was most intense in the 0–120 feet range, particularly in depths less than 40 feet.

Shrimp noise gradually tapers off with increasing depth. Near North Rock (Stations 25 and 26) the level of shrimp noise was relatively high. In deeper water, about a thousand feet northwest of North Rock (Station 28), shrimp appeared to be less numerous (as indicated by fewer snaps per second). A little farther off the edge of Bermuda Bank (Station 27) at 360 feet, no snapping shrimp noise was heard. Deep-water forms do occur, but not in sufficient numbers to produce the crackling sound typical of shallow water. In general, if bottom conditions are favorable, the relative abundance of snapping shrimp varies inversely with depth.

Bottom Conditions

Snapping shrimps occur mainly on substrates which afford shelter, such as rocks, coral, plant growth, or litter; only rarely on sand and mud bottoms. Many species live in association with other organisms. *Synalpheus* species are very numerous in large sponges (Coutière, 1910; Goode, 1878; Herrick, 1886; Hay and Shore, 1915–1916; Pearse, 1934, 1950). Snapping shrimps have been found living in burrows made by annelids or mollusks (Coutière, 1899), clinging to crinoids (Coutière, 1909), as commensals with tube worms (MacGinitie, 1935; Banner, 1953), anemones (Clarke, 1955), and ascidians (Lebour, 1938).

The Bermuda data indicate that snapping shrimps occur in great numbers only when sheltering materials are present. At almost every station where snapping shrimp noise was heard, the substrate consisted either of rock or coral. Conversely, at almost all stations with broken substrates (and less than 210 feet depth), snapping shrimp noise was heard. Apparent exceptions were Stations 7, 8, and 9, at the mouth of Castle Harbour. Much shrimp noise was heard at other stations in the area, and it would be expected that the noise would also have been heard at Stations 7, 8, and 9. Although no snaps were recorded, shrimp may have been present and their noise may have been cancelled by the great amount of water noise caused by the rough seas encountered at the time of recording. Stations with rocky coral bottoms had the highest level of shrimp noise.

In Bermuda the "sand" is not silicious but calcareous. In fact, at least 90% of all bottom deposits is composed of calcareous material (Bigelow, 1905). The south shore of Bermuda abounds in fine calcareous sand. As is to be expected, no shrimp noises were recorded near the south shore (Stations 43–45 and 54). Similarly, Johnson *et al.* (1947) and Shishkova (1958) never heard shrimp noise in areas with a sandy bottom.

At the deep Challenger and Argus Bank stations, where single snaps were not very numerous, snaps that were recorded on the vibragrams frequently occurred in clusters (Fig. 9). Although no snaps were recorded during the random time interval, occasional "volleys" of shrimp noise were heard. These bursts of snaps probably originated from groups of shrimps hiding under occasional cobbles.

Temperature

Slight temperature changes do not appear to have any great effect on snapping shrimp activity, but prevailing temperature determines their distribution. Their numbers dwindle as the water becomes colder. Specimens kept in glass aquaria survive well as long as the water temperature remains between 14 and 24° C. They can stand high temperatures and salinities and long periods without food. One specimen lived for months without food in an aquarium where the temperature reached 27° C. and the salinity was 45% (A. B. Williams, personal communication). They are more sensitive to low temperature.

An interesting situation described by Boden (1952) occurs on the edge of the Bermuda Bank. A convection current system exists where warm water of the lagoon flows over the bank until it converges with the surface of the colder ocean water. As the warm water is cooled, it sinks and the current is set up. In this way most of the warm lagoon water is returned to the lagoon. Plankton is more abundant in the lagoon, but much of it spills over the bank by means of this current system. Because larval forms of most species of snapping shrimp are planktonic, it is possible that shrimp larvae may be carried over the bank. This may explain why some shrimp noise was heard at the stations near the bank (Stations 28, 48 and 49). Snapping shrimps are probably fairly abundant on the bank due to the overflow of warm water from the lagoon.

Snapping Shrimp Spectrum Contrasted with Water Noise Spectrum

The main components of water noise occur in the frequency range 0–5 kc, as shown in Figure 10. The spectrum of water noise is shown by the dashed line in Figure 16, which represents an average of the intensity values at each 1-kc interval for each of the 17 stations where only water noise was heard. The graph shows that water noise decreases fairly regularly with increasing frequency. At frequencies above 8 kc the contribution of water noise to ambient noise is negligible.

Figure 11 is a vibragram of shrimp noise west of Flatts Inlet, where water noise was at a minimum (sea state 1). The vibragram is darkest in the frequency range 2–7 kc, indicating that shrimp noise is most intense in this range. Each spike, which represents an individual snap, exceeds the range of this vibragram. In Bermuda, shrimp noise completely dominates water noise from 2 to 5 kc and to a lesser extent below and above this range (Fig. 16). Everest *et al.* (1948) and Knudsen *et al.* (1948) also found that above 2 kc shrimp crackle is the major source of ambient noise. Using a Pimanov analyzer, Shishkova (1958) found that shrimp noise was greatest between 3 kc and 7.2 kc, but that the entire frequency spectrum of shrimp noise extends from 2.5 to 14 kc.

Unlike water noise, which decreases with regularity from 0 kc, there is a slight decrease of shrimp noise to 1 kc, then a slight increase to 2 kc, followed by a gradual decline with increasing frequency. This slight decrease of intensity at 1 kc appeared on many of the sections made (as in Figure 8) and is characteristic of snapping shrimp noise in Bermuda.

The average point of highest intensity of Bermuda shrimp noise is 2.2 kc, a point at which the relative intensity is 4.7. The point of highest intensity varied tremendously from station to station. Since shrimp noise level gradually decreases as the distance between bed and hydrophone increases (Everest *et al.,* 1948; Johnson, 1948), this variation in intensity was probably the result of differences in this distance.

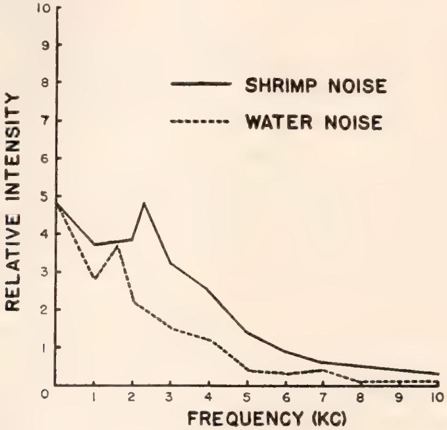


Figure 16. Spectra of shrimp and water noise at Bermuda.

The most reliable way to differentiate between the two types of noise is by noting the total frequency range. Snapping shrimp noise extends to frequencies of over 15 kc, but water noise is confined to the lower frequencies. Shrimp noise also differs from fish noise and spiny lobser stridulations, which are most intense at low frequencies (Loye and Proudfoot, 1946; Dobrin, 1947; Moulton, 1957).

Variability in Shrimp Spectra

A broad peak somewhere above 2 kc is characteristic of snapping shrimp spectra, but the frequency at which noise is most intense, as well as the degree of intensity, varies in different areas. Sound surveys in widely separated areas along the eastern and western coasts of the United States and in the Pacific Ocean have yielded a variety of shrimp noise spectra (Everest *et al.,* 1948). The point of highest intensity is different for each spectrum. Everest *et al.* found that in the San Diego Yacht Harbor, California, shrimp noise was most intense at about 2 kc, but in the Florida-Bahamas area it reached a peak at 10 kc. Analysis of shrimp noise in the Bimini area of the Bahamas, recorded during the summer of 1956 in conjunction with a study of the acoustical behavior of some fishes in this area (Moulton, 1958), revealed that the shrimp noise is most intense in the frequency range 3–8 kc (Fig. 12). The main intensity band was about 1 kc higher than in the Bermudas (compare Figures 12 and 11).

Everest *et al.* attributed some of the differences among the spectra which they obtained to differences in the measuring equipment employed. However, it is probable that variability of shrimp spectra is an inherent one and not due entirely to the use of different equipment. One of our recorders was employed both in the Bermudas and in the Bimini area, yet different spectra resulted with the use of the same instrument. This 1-kc difference in the spectra is more likely due to differences in the genera and species of predominating shrimp inhabiting the two areas and, more important, differences in their habitats. In the Bermudas snapping shrimps preferably occupy rock crevices. This kind of habitat tends to produce an echo effect, which is reflected in an intense band at the lower frequencies. In the Bimini area, most of the species of snapping shrimp (predominately *Synalpheus* species) inhabit the channels and pores of enormous sponges. This tends to result in a higher-pitched crackle. Thus, the echo effect is greater in Bermudian waters and this is presumably what causes the band of greatest intensity to be 1 kc lower than at Bimini. This resonance effect was also present when snaps of animals in aquaria were recorded. A water-filled aquarium provides excellent conditions for the recurrence of echoes.

Diurnal and Seasonal Variations

Studies by others demonstrated a diurnal variation in intensity of snapping shrimp noise, which increases slightly at night. Shrimp noise is 2 to 5 db higher at night than in the daytime, and it reaches a peak shortly after sunset and just before sunrise (Johnson *et al.*, 1947; Johnson, 1948). Knudsen *et al.* (1948) found that the peak in noise level is 3 to 4 db above the daytime level. A slight increase in activity of Bermuda populations during early evening was indicated (compare Stations 22 and 23). Animals other than snapping shrimp may indirectly cause an increase in noise level at this time by wandering about more then, thus inducing the shrimp to more snapping.

There is no significant seasonal variation in shrimp noise, at least in regions where the seasonal variation in temperature is small. Studies in the San Diego region, where water temperature remains relatively constant, showed no great fluctuations in noise level during the year (Johnson, 1948). Although no study of annual variations could be made in Bermuda, it is probable that in this region, as well as in all other areas where there is little seasonal variation in water temperature, there is little annual variation in shrimp noise. However, in places where there is a fairly large variation in water temperature, it is possible that temperature changes may have an effect on shrimp activity during the year.

Characteristics of Single Snaps

Individual snaps recorded in the laboratory were analyzed in order to compare them with the records obtained under natural conditions and to note any generic or specific differences in the spectra.

Individual snaps as they occur under the sea are essentially the same as those produced under laboratory conditions, but because of the great numbers of shrimp present, conditions under which the sounds are produced, and interfering water noise, differences in the spectra do exist. Where relatively few snapping shrimps occur and where water noise is at a minimum, the spectrum of a single snap is quite similar to that produced by an animal under laboratory conditions.

Recordings of *S. minus* snaps were made at the Bermuda Biological Station. The specimen was put in a wooden float (1 × 2 ft.) placed in sea water to a depth of 1 foot. A snap was recorded with the float 4 feet from the hydrophone (Fig. 13). Another snap was recorded when the float was 6 inches from the hydrophone, and still another with the specimen held in the hand 6 inches from the hydrophone. The spectra of these three snaps (Fig. 17) are almost identical. Thus, recording conditions do not significantly alter the spectrum of the snap. The main components of the *S. minus* snap lie in the frequency range 0–5 kc.

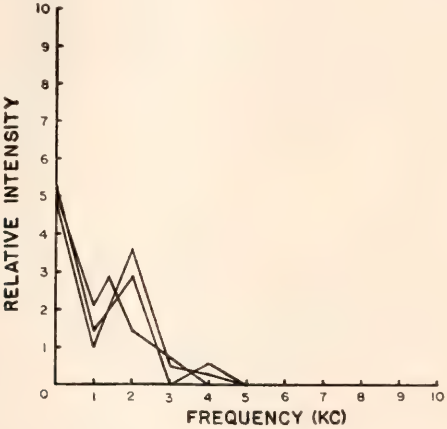
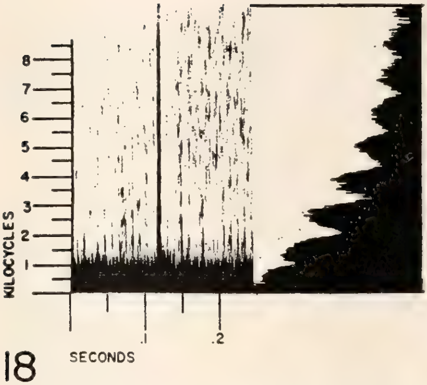


FIGURE 17. Spectra of three single *Synalpheus minus* snaps.

Synalpheus species are known to be prolific in the Bahamas and probably are the principal sound producers in this region. The laboratory records (Fig. 13) indicate that the point of highest intensity for *Synalpheus* lies in the range 1–2 kc, but the field records (Fig. 12) are most intense from 4 to 7 kc. When *Synalpheus* is not occupying a sponge pore, as in the laboratory recordings, its snap is most intense in the lower frequencies. But when, as in the waters of Bimini, it occupies its natural habitat within a sponge pore, the snapping noise becomes higher pitched, *i.e.*, its components are most intense at higher frequencies. This seems to indicate that variability in shrimp spectra around the world is to a greater extent the result of differences in habitat rather than differences in anatomy of the shrimp's claw.

Sounds produced by species of *Alpheus* were also recorded and analyzed. Figure 18 is a vibragram and section of a single snap from *A. armatus* recorded at Bimini. This *A. armatus* snap is characterized by a narrow spike extending to



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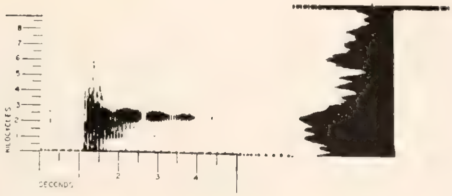

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Figure 18. Vibragram and section of a single *Alpheus armatus* snap.
Figure 19. Vibragram and section of a single *Alpheus heterochelis* snap.

the higher frequencies. The section exhibits a "step-like" pattern. It is not known whether this pattern is peculiar to *A. armatus;* the recording is unique.

Single *A. heterochelis* snaps were recorded and analyzed at Bowdoin College. Two snaps from two different specimens, one rather large in size (4 cm. long) and a smaller one (2 cm. long), were recorded, one with a contact microphone and the other with a hydrophone. The specimens were placed in an aquarium with a total water volume of 89 cubic inches. The spectra of these snaps appear in Figure 20. There is some variation (probably due to the fact that sections cannot all be made at corresponding time instants with great precision), but varying size of specimen or type of recording microphone does not seem to alter the spectrum substantially.

Figure 19 is a vibragram of an *A. heterochelis* snap in the 89-cubic inch aquarium, using a hydrophone. This snap is not in the form of a narrow spike as under natural conditions but covers a greater time interval, especially between

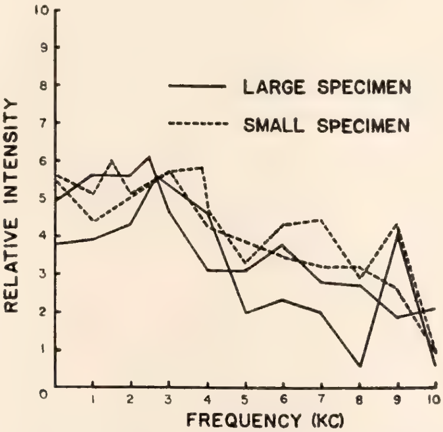

Figure 20. Spectra of four single *Alpheus heterochelis* snaps.

2 and 3 kc. This expansion is due to the echoes bouncing off sides of the aquarium and surface of the water. There is good evidence that snapping shrimp sounds are reflected from the water surface. Using a cathode-ray oscilloscope Everest *et al.* (1948) obtained oscillograms of individual snaps which show the sound wave itself and the echo, which is of smaller amplitude and opposite phase than the direct component. Because of echoing the sound seems to be much louder. This particular snap was easily heard by the unaided ear across the room.

It was found by increasing the frequency range to 704–70400 cycles per second that the *A. heterochelis* spectrum covered 52 kc. Johnson (1948) says that shrimp noise goes up to 50 kc. Shishkova (1958) analyzed the snaps of a snapping shrimp in a vessel and found the frequency band to be from 1 kc to only 6.3 kc. However, she does not mention the genus of the snapping shrimp nor does she describe the vessel. The low frequency range suggests the shrimp was a species of *Synalpheus*.

The spectrum of *A. heterochelis,* then, is quite different from that of *S. minus*. The *Alpheus* spectrum, which is greater than 10 kc., covers a much greater frequency range than the *Synalpheus* spectrum, which terminates at about 5 kc (compare Figures 17 and 20). Another noticeable difference is the amplitude of the peak intensity. In both cases the snap is most intense in the frequency range 1–4 kc, but the maximum intensity of the *Alpheus* snap is twice that of the *Synalpheus* one. Everest *et al.* (1948) noted some generic differences of wave shape between *Alpheus* and *Synalpheus* impulses. It seems clear that because claw structure of the two genera differ markedly, the sounds they produce would also be noticeably different. The structural pattern is uncertain. Whether dependable identifications can be made by sound analysis or not will be decided through further investigation.

Significance of Snapping Shrimp Noise

Shrimp crackle is useful as a tool in marine ecology. Information as to distribution of snapping shrimps can be obtained much more efficiently by this method than by laborious dredging, as Johnson (1948) pointed out. It is useful in determining depths and types of benthic environments. In the Bermuda area, at least, intense shrimp noise automatically indicates that the depth is less than 120 feet and that the bottom consists of coral, rocks, shells, or other sheltering materials. By spectral analysis it may be possible to pinpoint the type of substratum. Shrimp crackle may also be used indirectly to locate symbionts of the shrimp or other benthic animals inhabiting the same environment as the snapping shrimp. This use of shrimp noise has commercial implications. Detection of shrimp crackle with directional sound equipment may assist in finding commercial sponge concentrations, areas where the agar sea-weed *Gelidium* abounds, and fishing grounds (Johnson, 1948).

On the other hand, shrimp noise acts as a barrier in the study of underwater sounds. Because it is so intense and constant, it sets up a "curtain of sound" that makes it difficult to hear the sounds produced by other animals. Submariners are reputed to have masked engine and generator noise behind this acoustic curtain in wartime. But in any case, whether it is advantageous or not, it is there as the most persistent and most widespread biological noise.

The authors are indebted to Dr. Fenner A. Chace of the United States National Museum for assistance in the identification of specimens, to Dr. Austin B. Williams of the University of North Carolina for constructive criticism of the paper, and to Mr. Ernest R. Powell, who executed most of the drawings in this study.

SUMMARY

1. The snapping mechanism of snapping shrimps of the genera *Alpheus* (*Crangon*) and *Synalpheus* has been studied and the "crackle" produced by populations of these shrimp surrounding the Bermuda Islands has been analyzed.

2. Differences in chela structure between the two genera are enumerated. Earlier beliefs that the sound is produced primarily by contact of the calcified tips upon closure of the dactylopodite on the propodite are corroborated. It is concluded that the "suckers" in the articulation of the joint in *Alpheus* species do not serve as a cocking mechanism, and is suggested that suction develops in the living animal by depression of the membrane between the segments as the dactylus is raised. Overlapping exoskeletal knobs in the joint probably serve to hold the dactylus in place.

3. The structure and physiology of the chela musculature are as in the Crustacea generally. Both fast and slow contractions and an inhibitory effect are demonstrated. The functional significance of a doubly excitable system in this animal is discussed.

4. Analysis of snapping shrimp noise recordings at various locations around Bermuda shows that populations principally occur where sheltering materials are present and at depths of less than 120 feet. Prevailing temperature is also a limiting factor, since snapping shrimps occur mainly in tropical and subtropical waters.

5. In contrast to water noise, which is limited to the frequency range 0–5 kc, shrimp noise extends to frequencies of over 15 kc under natural conditions. Shrimp spectra from different areas are variable, due to differences in the predominating species inhabiting each area and, more important, to habitat differences.

6. There is a slight increase in shrimp noise at night but probably no significant seasonal variation at Bermuda, because water temperature remains relatively constant there throughout the year.

7. Under laboratory conditions generic differences in the spectra occur. The frequency range and amplitude of peak intensity of an *Alpheus* single snap are greater than in *Synalpheus*, as a result of the more powerful chela of *Alpheus*. Variation in recording conditions or size of specimen does not alter the spectrum substantially. The components of single snaps of specimens in aquaria extend to higher frequencies and cover a greater time interval than individual snaps under natural conditions because of greater echoing under laboratory conditions.

8. Snapping shrimp noise is useful in determining benthic environments and fauna.

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MARINE BIOL

A COMPARISON OF SALT LOSS RATE IN THREE SPECIES OF BRACKISH-WATER NEREID POLYCHAETES

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Different species of nereid polychaetes are able to regulate to varying degrees the salt concentration and osmotic pressure of the coelomic fluid in waters of low salinity (Schlieper, 1929; Beadle, 1937; Jørgensen and Dales, 1957; Smith, 1955). Hyper-regulation of coelomic salt concentration represents a steady-state in which losses of ions to the medium via body wall, nephridia, and gut are balanced by mechanisms of ion intake by body wall and/or gut. The tendency to lose body salts might be regarded as of negative adaptive value to a polychaete hyper-regulating in a situation of unchanging low salinity, since a high loss rate would impose a high load upon uptake mechanisms. On the other hand, the ability to lose salts rapidly might not be a disadvantage, and might be considered a favorable adaptation, in a polychaete subjected to a sudden lowering of environmental salinity, since by lowering the osmotic gradient the animal would be faced with a less severe problem of volume control and of disposing of excess water taken in osmotically. Indeed, it is likely that the process of effective volume control by urine output might contribute markedly to the lowering of internal salt concentration and so to the reduction of water intake to a point where mechanisms of volume control could be effective. Thus, while it might seem reasonable to expect to find a low tendency to lose salts in an estuarine or fresh-water polychaete, there are also reasons why an effective salt-losing mechanism could be of survival value in sudden exposures to low salinity. It has, therefore, seemed of interest to compare the overall tendency to lose salts in selected species of brackish-water polychaetes, in order to prepare for more detailed studies aimed at evaluating the separate roles of body wall, nephridia, and gut in this process. The present studies have been directed to the working out of methods for comparing salt-loss rates, and advantage has been taken of an opportunity to compare worms from certain widely-separated localities.

MATERIAL AND METHODS

Three species of brackish-water nereid polychaetes have been used in these comparisons:

1) *Nereis diversicolor* O. F. Müller, an extremely widespread species whose range includes brackish and estuarine waters in northwestern Europe, where it may penetrate into extremely low salinities, but does not live in fresh water except temporarily (Smith, 1955).

2) *Nereis limnicola* Johnson, a species found in estuaries on the west coast of North America, and in the fresh-water Lake Merced at San Francisco. It is closely related to *N. diversicolor* (Hartman, 1960; Smith, 1958), but is unique

in being viviparous. *Neanthes lighti* Hartman is a synonym. It should be noted that *N. diversicolor* and *N. limnicola* are closely related, even being considered conspecific by Hartman (1960), although other workers (Smith, 1958; Khlebovich, 1963) prefer to regard them as separate species. Both are referred to the genus *Neanthes* by Hartman, but this usage has not been uniformly accepted.

3) *Nereis (Neanthes) succinea* Frey and Leuckart, is widespread (Smith, 1963) both in Europe and America in brackish waters, but does not penetrate into such low salinities as does *N. diversicolor*. *N. succinea* is the same as the *Nereis limbata* Ehlers used in embryological studies at Woods Hole. It, too, is often referred to as *Neanthes*, especially in American usage. It should suffice for experimentalists that *N. succinea* stands somewhat apart from *N. diversicolor* and *N. limnicola*, and, unlike them, forms a reproductive swimming stage or "heteronereid." In the present study only its sexually immature "nereid" form has been used.

N. diversicolor has been studied at Kiel (Germany) near the mouth of the Baltic Sea, where the salinity approximates 50% sea water and at Turku (Finland), near the limits of its ecological range in the inner Baltic. *N. limnicola* has been collected from the relict fresh-water Lake Merced in San Francisco, and from the estuary of Walker Creek, emptying into Tomales Bay, California. *N. succinea* has been studied briefly at Kiel, and in more detail at Berkeley, using specimens taken in south San Francisco Bay, where salinities vary from 50% to 85% of sea water.

In order to have a standard basis for comparison of different nereid species in respect to their tendency to lose salts, it is necessary to set the experimental conditions rigidly, and the following procedures have been adhered to as closely as possible:

1. In order to assure the same osmotic or concentration gradient, worms are adapted before testing in sea water diluted to a chlorosity of 10 g./L., which is approximately equivalent to 55% of sea water and has a conductivity roughly that of 0.3 molar NaCl. At such a concentration, these worms are above the regulatory range and essentially conform to the chlorosity and osmotic concentration of the medium.
2. The test of salt loss rate is made immersing the worms (after a brief rinse) in a measured volume of glass-distilled or de-ionized water for one hour, at the end of which the conductivity of that water is measured and the salts lost calculated as the equivalent of NaCl or KCl. The volume of distilled water used was 1 ml. per 2.5 mg. wet weight of worm, and the concentration of salts lost was so low that recovery by active uptake could be disregarded. (Note: Metal-distilled water is toxic, resulting in permanent injury, and must be avoided.) The one-hour exposure in these experiments caused great swelling in *N. succinea*, but recovery appeared to be complete after return to the adaptational medium.
3. Since salt loss is undoubtedly taking place through external body surfaces as well as via nephridia and possibly gut, it is to be expected that it would be related both to body weight and to surface. The data obtained under the specified conditions have been plotted as straight lines on a double log plot, according to the common expression: Loss = a (Weight)b where "Loss" is the millimoles of salt lost per worm per hour; "Weight" is the wet weight in milligrams taken before

exposure to distilled water; "b" represents the slope of the line in the double log plot, and "a" the intercept at the y-axis when x = unity. Expression of results in this fashion permits comparison between species and populations differing in mean size and size range, as is essential in working with such animals, since it is generally impossible to collect worms of uniform or specified size in sufficient number, or to find populations of the same mean size in different localities. Considerable scatter has been found in these studies, which may in part be caused by variation in the amount of water held in the gut, although all worms were kept about a day in clean water to allow the emptying of sand and fecal material. Attempts to use dry weight did not, however, produce detectably less scatter.

4. Salt loss in distilled water should be carried out at a uniform temperature; this has unfortunately varied somewhat in different laboratories: the room temperature was 18–21° C. at Kiel; controlled baths at 18° C. in Turku, and at 15° C. in Berkeley, were used. Standard conductivity bridges (Siemens, Philips, and Industrial Instruments, Inc.) have been used. A blood-pipette conductivity cell was used at Kiel, a Philips dip cell at Turku and Berkeley. Conductivities have been expressed as equivalent millimoles of monovalent salt (NaCl or KCl) read from calibration curves constructed for the temperature and the cell used in each laboratory, and the salts lost by each worm in the known volume of water calculated.

It is clear that this type of comparison is based upon a highly unnatural type of exposure of the worm to minimum salinity. The tests are, in fact, a standardized osmotic emergency. The plunging of a worm into salt-free water not only results in salt loss but also water entry, and initiates responses of osmotic and volume regulation. The rate of salt loss declines with time; hence even an exposure as short as one hour gives too low a value for the rate existing at the start of the exposure; this fact is of lesser importance when comparing *N. diversicolor* and *N. limnicola,* since the drop in salt loss rate during one hour is not very great (*i.e.,* the loss curve does not markedly decrease in slope), but it is undoubtedly important in the case of *N. succinea,* in which the osmotic gradient is greatly lowered by swelling during one hour, so that the loss rate as calculated over one hour must be lower than the initial rate. This limitation has been accepted for this preliminary survey, since it represents an error on the conservative side.

In addition to experiments involving the standard test procedure indicated above, some experiments have been performed on worms adapted to sea water dilutions of different chlorosity (1, 5, 15 and 18.6 g./L.), and in some instances isotonic solutions of non-electrolytes have been used instead of glass-distilled water in an effort to clarify certain problems. These will be mentioned below, in context.

Experimental Results

a) Inter-specific comparisons of salt loss rates

In Figure 1 are curves for the salt-loss rates of the three species, each represented by two populations. Inspection indicates that *N. succinea* loses more salt under the conditions of this experiment than do the other two species, which, as

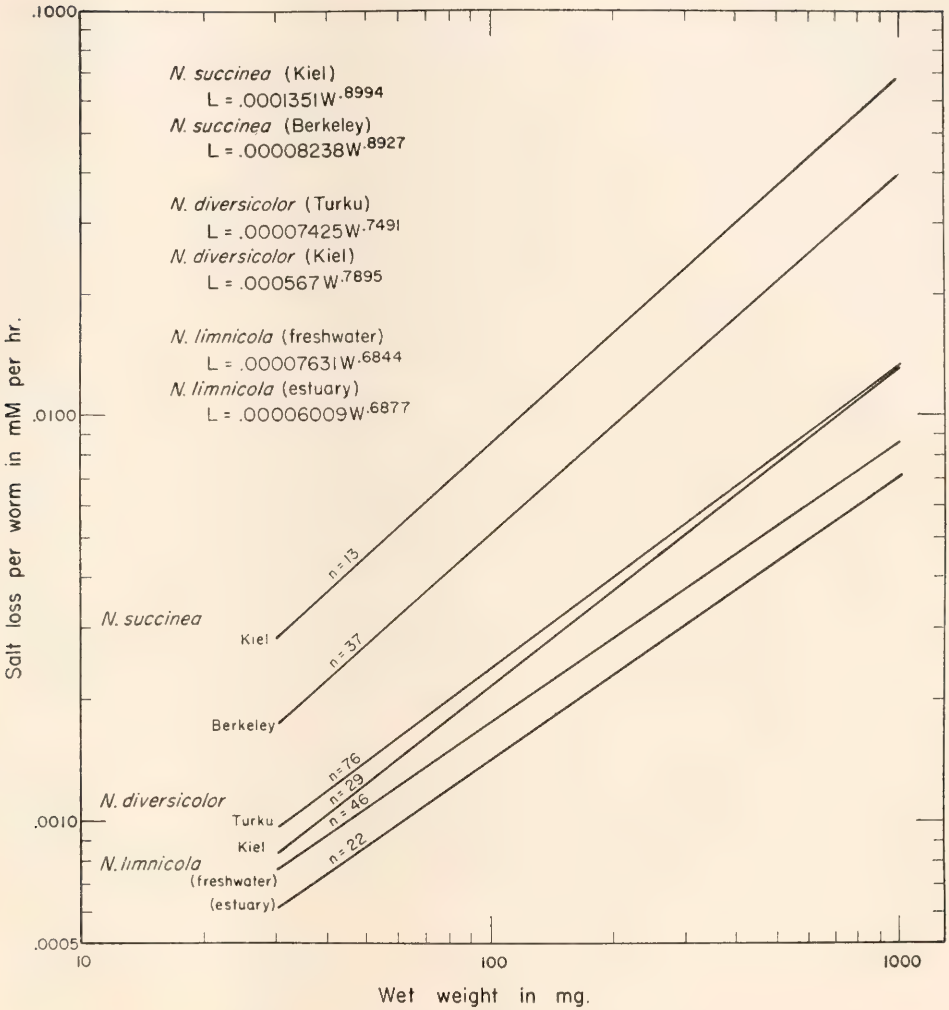

FIGURE 1. Salt loss per worm as a function of body weight under the standard conditions described in text.

expected, are more similar to each other. The salt losses per worm weighing one gram, as read from the curves, are:

N. succinea (Kiel)	0.068 m*M*/hr.
N. succinea (Berkeley)	0.039 m*M*/hr.
N. diversicolor (Kiel)	0.0129 m*M*/hr.
N. diversicolor (Turku)	0.0131 m*M*/hr.
N. limnicola (Lake Merced)	0.0086 m*M*/hr.
N. limnicola (Walker Creek)	0.0070 m*M*/hr.

The values for *N. succinea* show it losing 3 to 5.2 times as much salt per gram as *N. diversicolor,* which in its turn loses 1.5 to 1.9 times as much as *N. limnicola.*

For reasons mentioned above, the tendency of *N. succinea* to lose salts is actually greater than these curves indicate. The general height of these curves indicates that lessened salinity of the habitat is associated with a lesser rate of salt loss when the species are compared.

Not only do the salt loss curves differ in height between *N. succinea* and the other two species, but the slopes (b) are markedly greater in the case of *N. succinea* (0.899 and 0.893) than in the case of *N. diversicolor* (0.749 and 0.790) and *N. limnicola* (0.684 and 0.688). Values as low as 0.68 suggest that *N. limnicola*'s salt loss rate approximates the "surface rule." This may mean that this species does not change shape as it grows (a view that inspection verifies in a subjective way), and hence suffers the expected decrease in surface/volume ratio with increase in size. On the other hand, *N. succinea* seems to depart markedly from the "surface rule," and, although it does not attain a "b" value of 1.0, which would mean that it succeeded in keeping its surface/volume ratio constant during growth, its "b" value or slope of 0.89–0.90 suggests that it does change shape in such a way as to lessen the decrease in its surface/volume ratio during growth, a view subjectively confirmed by inspection of the conspicuously elongated posterior dorsal parapodial lobes developed by older and larger worms. Until a quantitative measure of surface area is available, this is the simplest explanation of the steeper slope of the salt loss curves of *N. succinea*. Since the curves for *N. succinea* would not cross those of the other two species unless extrapolated to unreasonably small weights, it is felt that the observed difference in height of the curves within the observed range of weights (roughly 30 to 1000 mg.) is the result of actual differences in outward permeability to salts rather than solely due to a greater surface/volume ratio in *N. succinea*.

Measurement of salt loss at short intervals shows that salt loss in these worms follows an expected course, dropping with time, as would be expected for two reasons, (1) that the gradient decreases because of salts passing out, and (2) that the gradient is lowered by dilution of the internal salts by water entering osmotically. The average percentages of swelling observed in the three species during the one-hour exposure to distilled water are:

N. succinea (Kiel)	50–60%
N. succinea (Berkeley)	57%
N. diversicolor (Kiel)	24%
N. diversicolor (Turku)	25%
N. limnicola (San Francisco)	10%

In the case of *N. succinea*, a net gain of over 50% the initial weight caused by water entry must have diluted the coelomic fluid enough to lower the salt gradient by at least half. At the same time, it may have had the effect of increasing the urinary output. Since the effect would have been simultaneously to reduce salt loss *via* the body wall and to increase it *via* the urine, these data do not permit one to state whether the recorded salt loss has been increased or decreased by the fact of osmotic water entry. An absolute value for urinary water output and salt content would be needed to answer this question, as well as information on the space occupied by the incoming water in the hour of exposure. Similarly, the lesser salt losses of *N. diversicolor* and *N. limnicola* cannot be attributed to their lesser degree of osmotic swelling, and we cannot say whether the smaller swelling represents a smaller amount of water entry, or a more effective disposal of the water as urine. The latter two species, as compared to *N. succinea:* (a) are clearly more effective volume-regulators, remaining active and normal in appearance during the exposure, whereas *N. succinea* becomes swollen and motionless (although capable of quick recovery); (b) are more conservative of salts in the sudden exposure to fresh water, although it is not apparent whether low outward salt permeability or effective salt retention in nephridia is involved; and (c) show a decrease in salt loss rate with increase in body size, seemingly having the advantage of the "surface rule" as a consequence of constant body shape during growth.

The curves of salt loss rate in Figure 1 are in the order of decreasing salt loss with decreasing salinity of habitat of the three species involved. *N. diversicolor* at Kiel (habitat chlorosity about 10 g. Cl/L.) and at Turku (habitat chlorosity about 3 g. Cl/L.) behave almost identically. *N. succinea,* although overlapping the salinity range of *N. diversicolor,* is characteristically in a more saline habitat and does not tolerate such low salinities. At present no explanation of the higher salt loss rate observed at Kiel can be offered. The number of worms used at Kiel was small, and the possibility of experimental error in making up the adaptational media is not excluded. The curves for the fresh-water and the estuarine populations of *N. limnicola* seem paradoxical in that the salt loss is greater in the fresh-water group. There are probably two reasons for this: (1) Studies of the level of chloride regulation in *N. limnicola* (Smith, 1959) have shown that the Lake Merced population regulates its coelomic chloride above the level in the Walker Creek estuarine population, and even holds it slightly above the medium at a chlorosity of 10 g. Cl/L.; thus the salt gradient for the fresh-water population may have been slightly higher. (2) Studies on volume control (Smith, 1959) likewise showed that the fresh-water population of *N. limnicola* consistently exhibited a lesser swelling and a faster return to normal volume after transfer from sea water of chlorosity 10 g. Cl/L. to fresh water. This difference in response was not eliminated by over 60 days of adaptation of fresh-water and estuarine worms to identical salinities in the laboratory. The fresh-water population of *N. limnicola* is able to eliminate water faster than its estuarine relatives; presumably the extra urine discharged, at least in the early stages of adjustment in distilled water, eliminates salts and contributes to the greater salt loss rate of the fresh-water population. Thus the greater salt loss rate is seen as an advantage in rapid adjustment to the osmotic emergency.

b) Salt loss in N. diversicolor *as a function of the gradient*

In the inter-specific comparison just made, *N. succinea* showed a higher "b" value, which is thought to be a consequence of an increase in relative body surface during growth, causing a departure from the "surface rule." However, it might be asked if this high value of "b" is not in some way related to the very great swelling endured by *N. succinea* during the hour in distilled water. Although this view seems unlikely because the surface/volume ratio would be *decreased* by swelling rather than increased (note that the surface of a swelling nereid can unfold like an accordion, rather than stretching like a balloon), it may be tested experimentally by comparing the "b" values of the salt loss curves obtained under conditions in which the amount of swelling is varied. This experiment was done using *N. diversicolor* at Turku. Groups of worms were adapted to dilutions of sea water of chlorosities 1, 5, 10, 15 and 18.2 g. Cl/L. prior to the standard one-hour exposure in distilled water. In Figure 2 are plotted the percentages of swelling as a function of the adaptational chlorosities. As expected, worms with the higher salt gradient swelled more. Note that worms adapted to 1 and 5 g. Cl/L. behaved in this respect rather similarly; these chlorosities lie in the "regulatory range," so that the internal concentrations are much the same. The worms adapted at 1 g. Cl/L. even showed a decrease in volume when placed in distilled water. This effect has often been noted, and probably reflects an increase in urinary output caused by increased activity and muscular tension, as postulated by Beadle (1937) in relation to volume control. Likewise, in Figures 2 and 3 it may be noted that salt loss in the worms adapted to 1 and 5 g. Cl/L. is almost identical, suggesting that the amount of salt lost in these experiments is a function of the gradient existing between interior and the medium. However, the "b" values of the set of curves in Figure 3 lend no support to the idea that the high "b" values for *N. succinea* could have been a result of excessive swelling. Rather, they suggest that volume increase merely depresses the surface/volume ratio. The low "b" values of 0.44 and 0.53 seen in the worms from chlorosities of 18.2 and 15 g. Cl/L. lend credibility to the supposition that the surface of a swelling nereid does not increase in area but merely unfolds as the worm takes in water. On this view, the extensive surface developed by a large *N. succinea,* even in the "nereid" form,

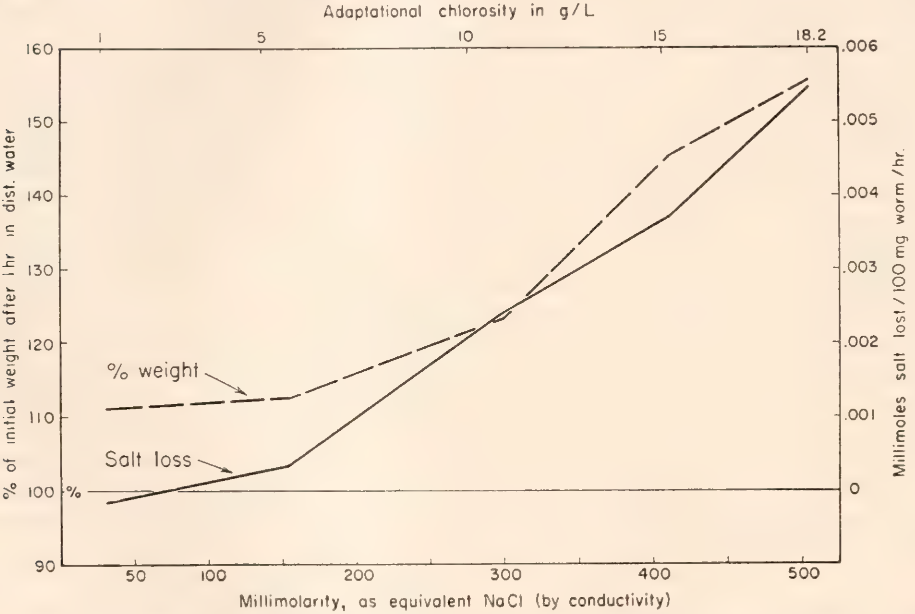

FIGURE 2. Relationship between the percentage of swelling (left-hand ordinate), the amount of salt loss per 100 mg., from curves in Figure 3 (right-hand ordinate), and the chlorosity and approximate millimolarity (equivalent NaCl) of the media to which *N. diversicolor* were adapted prior to one-hour exposure to de-ionized water.

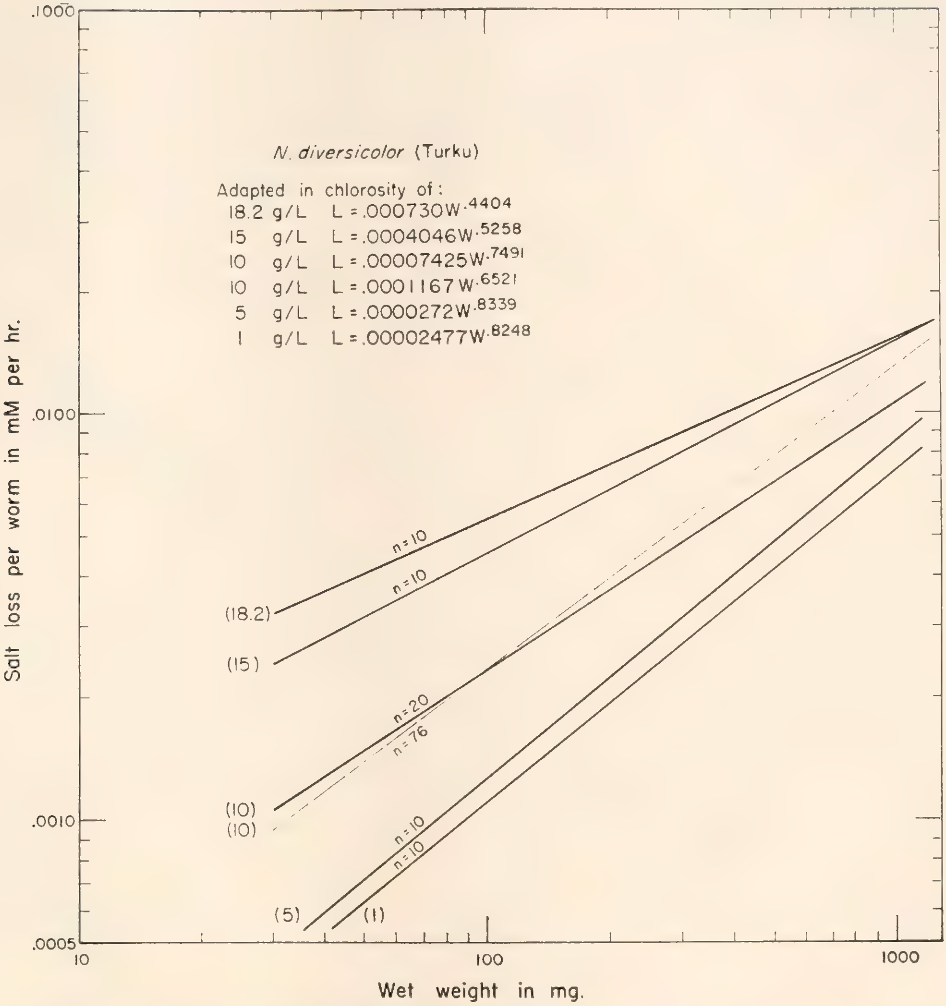

FIGURE 3. Salt loss per worm as a function of body size following adaptation to different chlorosities of diluted sea water. Note the differences in slope associated with the osmotic gradient and degree of swelling in the standard exposure to distilled water (see also Figure 2).

becomes a factor of importance in determining its lesser ability to retain salts and resist swelling when exposed to low salinities.

c) The effect of preventing osmotic water-entry upon salt-loss rates

The results of adapting *N. diversicolor* to various concentrations of sea water before the acute test of salt loss have indicated that the rate of salt loss to distilled water is a function of the salt gradient. However, since this gradient must be lowered by water entry during swelling, it was thought that if swelling could be prevented by the use of isotonic salt-free solutions rather than distilled water in

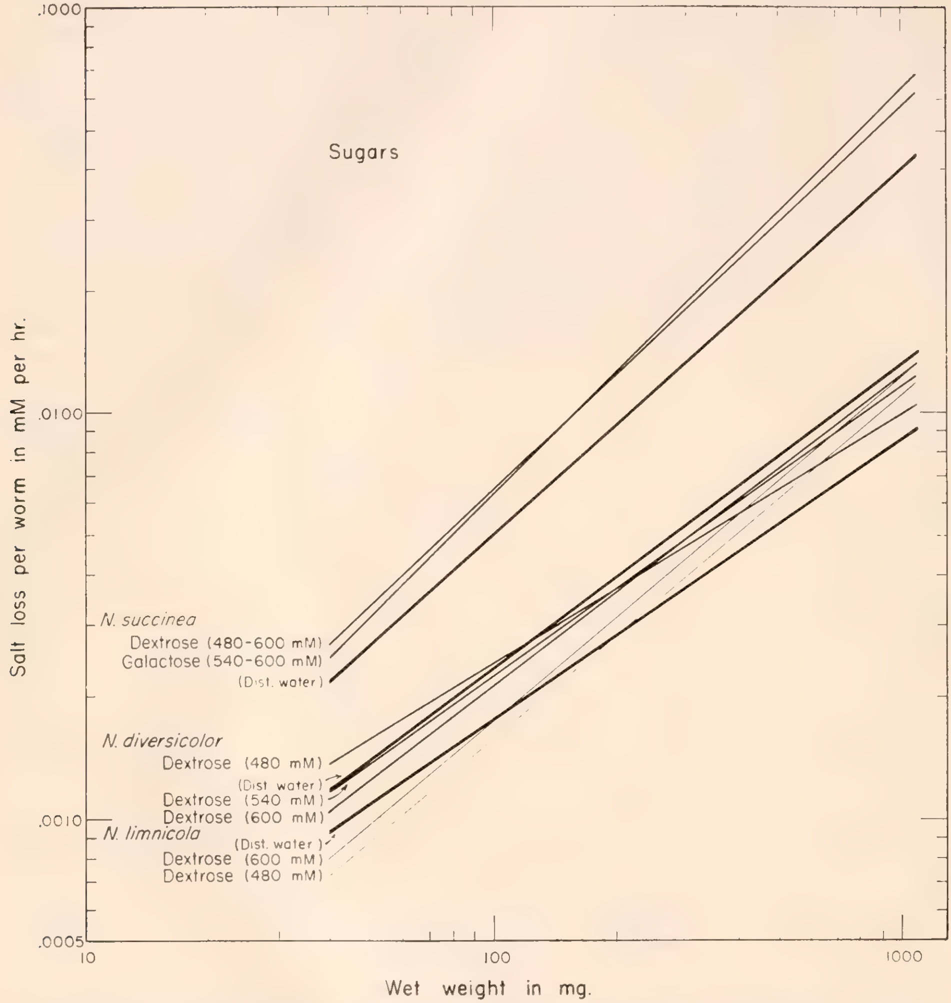

FIGURE 4. Typical examples of salt loss into isotonic (600 mM) or moderately hypotonic sugar solutions after adaptation to sea water of chlorosity 10 g./L. Least change of volume usually in approximately 90% of isotonic value. Control curves (distilled water) are from Figure 1. Loss is slightly increased from *N. succinea,* but no effect is demonstrable in *N. diversicolor* or *N. limnicola.* All curves from conductivity measurements.

the acute salt-loss tests, the salt loss rate might thereby be increased. Accordingly, a number of experiments have been made in which isotonic solutions of dextrose, sucrose, or galactose have been substitute for distilled water in standard salt-loss determinations. The results (Fig. 4) reveal no increase in salt loss rate in *N. diversicolor* or *N. limnicola,* species in which the extent of osmotic swelling in distilled water is slight. It has been consistently noted that a sugar solution isotonic to the adaptational sea water causes some loss of volume during the exposure; this volume loss is attributed to output of urine while osmotic water inflow is stopped. Commonly, such worms swell when returned to the original media, suggesting that a concentration of the body fluids has occurred; this hypothesis would seem to require that the urine be hypotonic to the body fluid. Sugar solutions of 85–90% the osmotic pressure of the adaptational sea water caused very little volume change, suggesting that they allowed a net osmotic inflow equal to the urinary output.

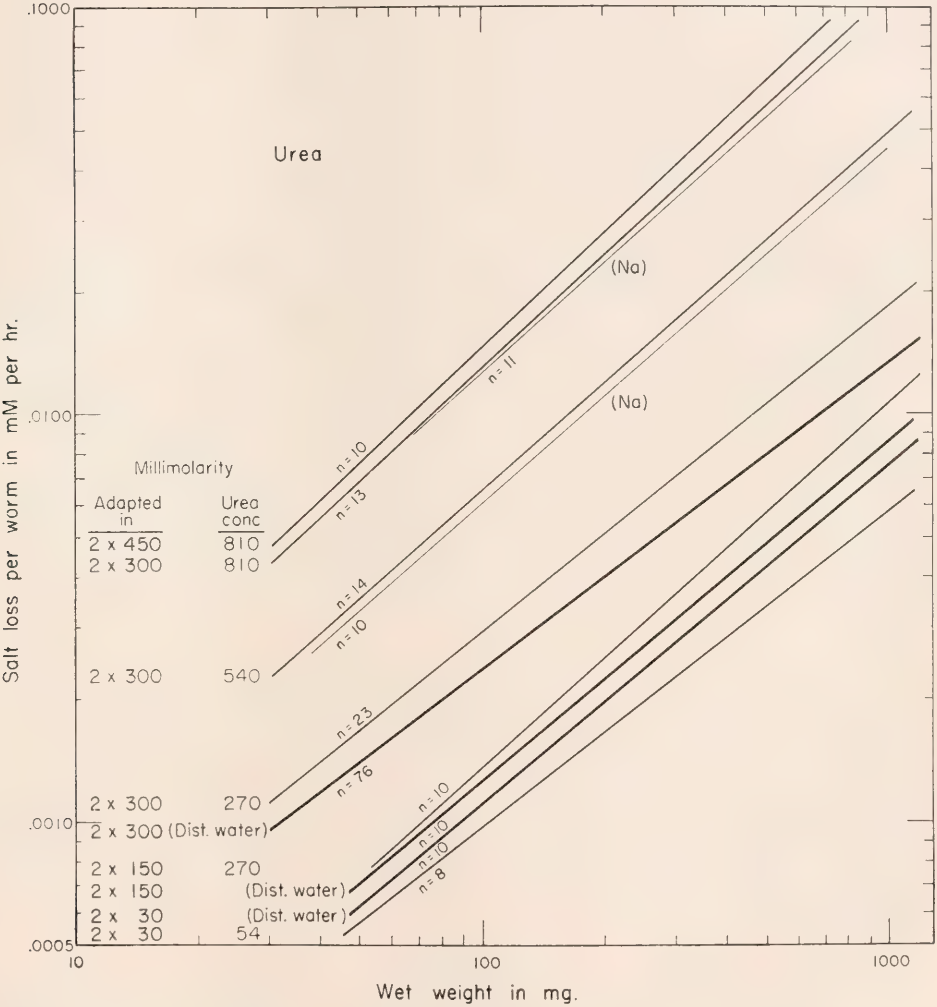

FIGURE 5. Salt loss into salt-free urea solutions, after adaptation to sea water diluted to 15, 10, 5 and 1 g. Cl/L., equivalent to NaCl concentrations of 450, 300, 150 and 30 mM, urea molarity set in relation to 2 × salt molarity. Note especially that salt loss level after adaptation to 10 g. Cl/L. (approximately milliosmolarity 2 × 300) increases with urea concentration, probably as an injury effect. Thin lines marked (Na) are calculated as 2 × the Na-millimolarity as determined by flame photometry; the rest by conductivity.

In the case of *N. succinea,* such sugar solutions seemed to cause a slight increase in salt loss rate, probably attributable to the maintenance of the salt gradient as a result of preventing dilution of the body fluids. The effect is not, however, very great. It seems to suggest that in *N. succinea* the maintenance of the salt gradient increases the outward passage of salts, and since the urine flow is probably not increased, this increased salt loss may be *via* the body wall. This latter point is, however, only inferred, not proved.

Surprisingly, even though salt-free sugar solutions prevented swelling and increased the salt loss only slightly (*N. succinea*) or not at all, the final condition of all three species after an hour in such solutions was clearly worse than in distilled water. They became motionless and often showed protruded pharynges; the anterior part of the body might be contracted and the head region flexed dorsally. Although recovery seemed complete, the impression was unavoidable that the sugar solutions were not physiologically neutral, and that the prevention of swelling was not free of side effects, the nature of which is not known. Possibly there is some penetration or active uptake of sugar into the body, although the fact that dextrose, galactose, and sucrose all had similar effects renders this explanation unlikely. Possibly the reduction of normal water fluxes leads to an ionic imbalance. Although the effects described merit further study, the use of isotonic solutions of non-electrolytes did not seem to contribute to clarification of the comparative performances of the species under study, and was discontinued.

Isotonic solutions of urea were also tested, upon *N. diversicolor* only. In all cases, urea solutions increased the salt loss rate, but here the rate of salt loss seems related to the concentration of urea applied, rather than to the salt concentration gradient (Fig. 5). The results are explicable on the basis of increased outward permeability to salts caused by the urea in proportion to its concentration. The worms recovered from short exposures, but exposures of an hour or longer were clearly deleterious. Since experiments with urea evidently involved damage to the body surface, they also were discontinued.

It is a pleasure to be able to express here my gratitude to Prof. Dr. Carl Schlieper of the Institut für Meereskunde, University of Kiel, in whose laboratory I was privileged to commence this study. I thank him for his help and hospitality, and thank also Dr. Jurgen Flügel, Dr. Kurt Hohendorf, and Frl. Ingrid Schäfer of that laboratory for their assistance. Work in Finland was made possible by a Fulbright visiting professorship in the Zoology Department, University of Turku, and was aided by the kind help and advice of Dr. Kari Lagerspetz and the technical assistance of Mr. Juhani Kuula, with support of a grant from the U. S. National Science Foundation.

SUMMARY

1. A standardized method is presented for measuring and comparing the total salt loss rates of different species of brackish-water polychaetes acutely exposed to an osmotic emergency situation.

2. *Nereis* (*Neanthes*) *succinea* shows a high salt loss rate in comparison to *Nereis diversicolor* and its close relative *N. limnicola*. These rates decrease in proportion to the degree of salinity lowering tolerated by these species in their natural habitats.

3. In *N. diversicolor,* the rate of salt loss is proportional (under the standard conditions) to the concentration gradient between body fluid and outside medium.

4. In *N. limnicola,* the tendency of the fresh-water population to have a higher salt loss rate than an estuarine population is seen as a result of a higher level of salt regulation and a more effective volume regulation by urinary output.

5. The slopes of the salt loss curves suggest that *N. succinea* changes shape during growth in such a way as to lessen the expected decrease in its surface/volume ratio, and thus departs from the "surface rule." *N. limnicola* (and, to a lesser extent, *N. diversicolor*) does not seem to change shape during growth and follows the "surface rule" in respect to rate of salt loss.

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HISTOLOGY OF THE REPRODUCTIVE SYSTEM OF THE SOFT-SHELL CLAM (MYA ARENARIA)

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In many kinds of experimental work with marine organisms, a means of inducing them to spawn under laboratory conditions is often essential. Although many species of lamellibranch molluscs spawn readily in response to suitable stimuli, the soft-shell clam (*Mya arenaria*) is more refractory, responding poorly and erratically to the commonly used techniques of stimulation. To gain a better understanding of the spawning mechanism in *Mya,* a detailed study of the morphology and histology of its reproductive system was undertaken. Although the anatomy of *Mya* was described in some detail by Vlès (1909), only a few lines were devoted to the reproductive organs. Coe and Turner (1938) described the reproductive system more thoroughly, but less attention was given to the accessory organs than to the gonad itself. Furthermore, the discussion of the system was presented from a developmental viewpoint and dealt primarily with immature animals. The purpose of the present paper is to describe the morphology and histology of the reproductive system as a whole, with particular emphasis on structures other than the gonad.

Because *Mya* differs from many other species in its responsiveness to artificial spawning stimuli, any peculiarities in the morphology of its reproductive system must be examined in the light of their possible relationship to spawning activities. For this reason, comparative studies were made of two additional species: the surf clam, *Spisula solidissima,* and the hard clam, *Mercenaria mercenaria,* to determine whether Mya differed significantly in the morphology of its reproductive system. Observations on the spawning activities of these species were also made to learn what aspects of function might correspond with peculiarities in structure.

METHODS

The information presented in this paper was based on the following sources:

(1) Several hundred histological preparations of the gonad tissue of *Mya arenaria,* representing all seasons of the year, were examined.

(2) Gross dissections and examinations were made on fresh specimens of *Mya arenaria, Mercenaria mercenaria* and *Spisula solidissima.*

(3) Living specimens of these three species were held in aquaria and their spawning activities observed.

(4) Serial sections of tissues fixed in Bouin's fluid were made through the region of the genital papillae of 13 randomly selected specimens of *Mya,* 5 of *Spisula* and three of *Mercenaria.* These sections were stained with haematoxylin and eosin and mounted on slides.

Photographs were made of various structures in the histological preparations and reconstructions in the form of sketches were made to clarify the spatial relationships of the structures.

Spawning of the living specimens was artificially induced by a cyclic fluctuation in water temperature, or in a few cases by injecting 1 ml. of 0.1 N ammonium hydroxide into the gonad through the mantle strap.

RESULTS

In general arrangement, the reproductive system of *Mya* is similar in both sexes. The gonads are paired organs consisting of highly ramified tubules bearing numerous terminal and lateral alveoli. In a well-nourished, ripe individual these alveoli are closely packed and fill much of the visceral mass. The tubules of each gonad, by a series of confluences, eventually merge into a pair of gonoducts (Fig. 1) which lead caudad and dorsad towards the region of the genital apertures. The apertures are situated on tiny papillae located on either side of the dorsal apex of the visceral mass (Fig. 2), just anterior to the foot retractor muscle. According to Vlès (1909), the gonads remain isolated from one another throughout the system, so that ink injected into one of the genital apertures will permeate the genital organ on one side of the clam only. While this mutual isolation seemed to be true in some of the individuals which I examined, in most specimens the gonoducts were joined in a common chamber or vesicle (Figs. 1 and 3), lying between and slightly anterior to the genital apertures. The vesicle is often bilobed, suggesting a tendency toward separation. Leading caudad and laterally from this vesicle are two short ducts (referred to henceforth as the "terminal gonoducts") which lead to the genital apertures. The apertures, opening into the dorsal pallial cavity, are in the form of slits curving from the termini to the lateral sides of the papillae.

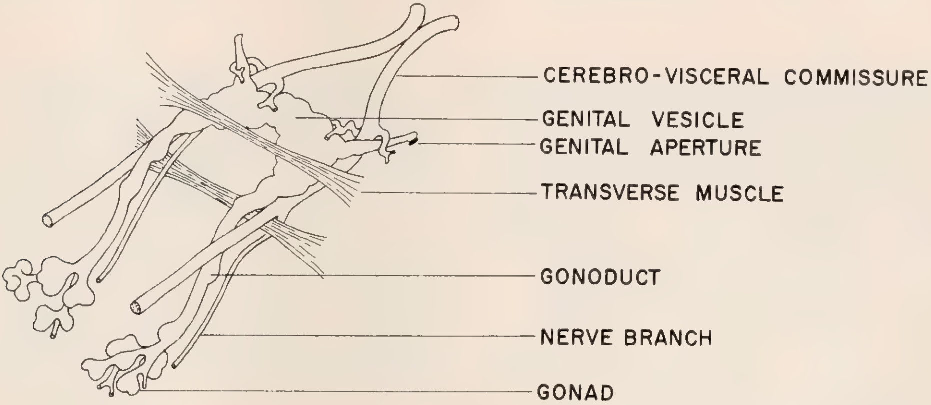
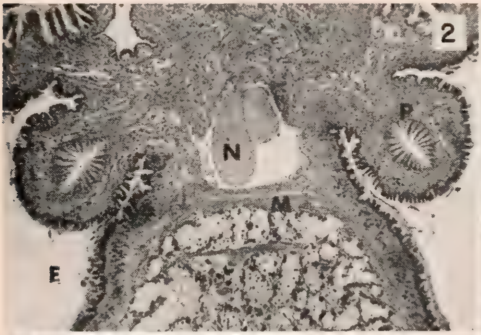


FIGURE 1. Semi-diagrammatic reconstruction of the reproductive system and associated structures in *Mya arenaria*.

The paired nerve trunks of the cerebro-visceral commissure pass directly under the terminal gonoducts, giving off many small branchlets which invest the connective tissue in this region. A larger branch from each of these nerve trunks follows the gonoduct from the genital vesicle deep into the gonad itself. The wall of the visceral mass contains abundant muscular tissue; connecting these walls transversely are numerous bands of muscle fibers which pass through the gonad.

The histological structure of the gonad alveoli has been described by Coe and Turner (1938). These alveoli consist of a thin basement membrane or wall surrounding a group of large, transparent follicle cells, which may fill the alveolus solidly, or may separate in the center to form a lumen. The gametes arise from the basement membrane between the peripheral follicle cells. The gonoducts and their branches resemble the alveoli in histological structure, except that the large, transparent cells tend to be more elongate, oriented perpendicularly to the basement membrane, and a lumen is always present. Gametes are also proliferated from the basement membrane of the gonoducts, although less abundantly than in the alveoli. In some specimens the transparent cells lining the gonoduct are reduced in size and appear to be disintegrating. Where this process is taking place, clusters of clear droplets appear in the lumen, suggesting holocrine secretory activity.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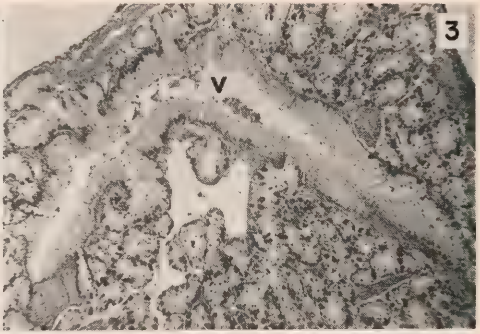
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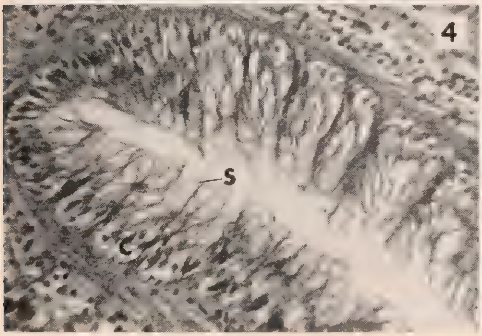
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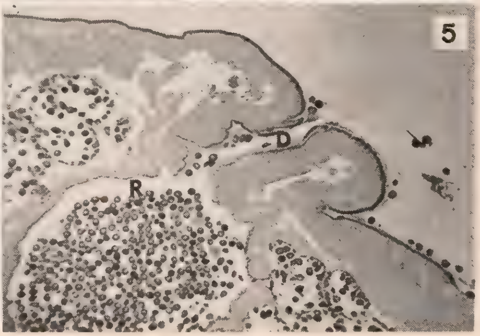
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FIGURE 2. Cross-section through the visceral mass of *Mya arenaria* at the level of the genital papillae. Papillae (P), cerebro-visceral nerve commissures (N), transverse muscles (M) and dorsal pallial chamber (E) are indicated. × 35.

FIGURE 3. Cross-section through the visceral mass of *Mya arenaria* at the level of the genital vesicle (V). × 35.

FIGURE 4. Cross-section of terminal gonoduct of *Mya arenaria* taken through genital papilla, showing both ciliated columnar (C) and non-ciliated "secretory" cells (S). × 300.

FIGURE 5. Longitudinal section through genital papilla of *Spisula solidissima,* showing terminal gonoduct (D) and enlarged portion of main gonoduct (R) used as reservoir for eggs. × 25.

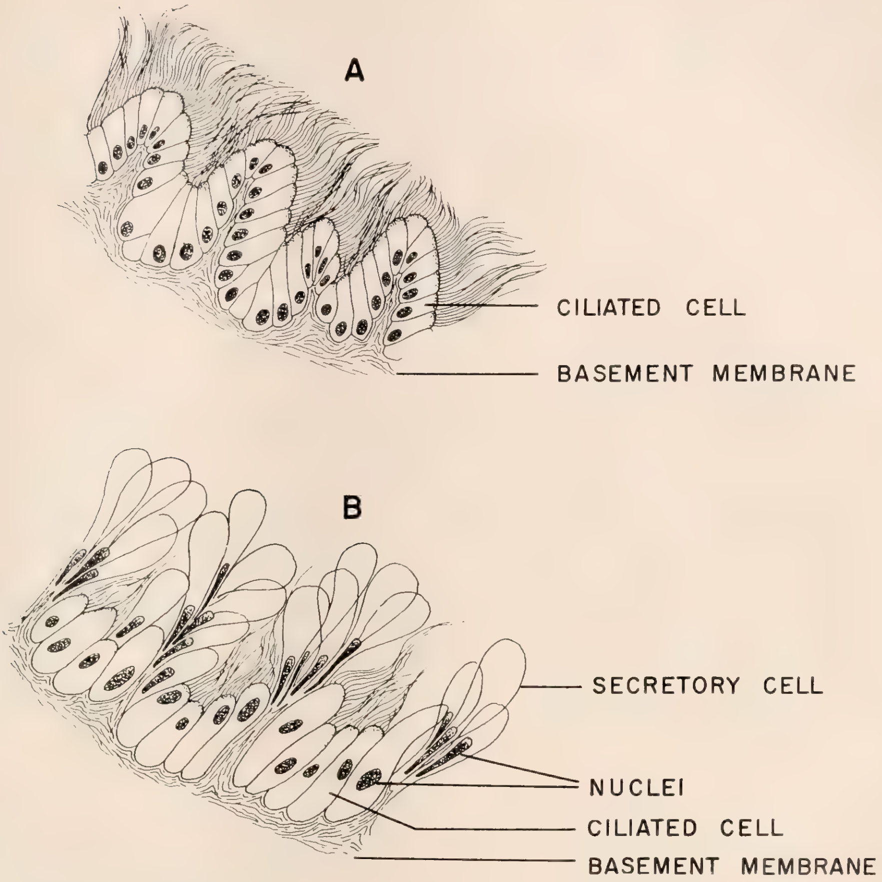

FIGURE 6. Camera lucida drawings, somewhat simplified for clarity, showing portions of the ciliated (A) and non-ciliated (B) epithelial tissues in the terminal gonoduct of *Mya arenaria*.

The tissue lining the terminal gonoducts deserves particular notice. The interior walls are wrinkled with longitudinal ridges of connective tissue upon which rests an epithelium of columnar and other cells, the nature and arrangement of which is variable. In the simplest case, all are ciliated columnar cells with prominent elliptical nuclei (Fig. 6A). The cytoplasm stains lightly with eosin, more intensely so near the ciliated ends of the cells. In a somewhat more complex arrangement, the longitudinal ridges may extend farther into the lumen to form thin folds rather than simple ridges. The ciliated columnar cells on these folds become or are replaced by elongated, non-ciliated cells with bulbous ends containing a granular cytoplasm. In what appears to be an advanced stage of this development, the bulbous ends become very prominent and more transparent, and groups of them on the crests and sides of the folds of connective tissue suggest, in section, clusters of fruit on a stalk (Figs. 4 and 6B). Groups of these specialized cells alternate with groups of ciliated cells, or may sometimes replace them entirely. Many intermediate arrangements between the extremes of simple ciliated columnar epithelium to the highly modified bulbous cells can be found in different individuals. The arrangement may differ between the two terminal gonoducts of the same individual.

The function of the ciliated cells is undoubtedly to assist in the extrusion of gametes from the genital aperture. That of the other cells is uncertain, but their appearance suggests a secretory function. Because of the variation in the arrangement and nature of this epithelial tissue, it is possible that a cyclic transition occurs in a manner similar to that in tissues of certain reproductive organs in higher animals. However, no relationship was apparent between the condition of the gonoduct epithelium and the sex or the stage in the seasonal reproductive cycle of the clams examined.

The reproductive systems of both *Mercenaria,* described by Loosanoff (1937a, 1937b), and *Spisula* are similar in many respects to that of *Mya.* One essential point of difference is the lack in the former species of the large follicle cells characteristic of *Mya.* Also, both *Mercenaria* and *Spisula* possess more abundant muscular tissue throughout the visceral mass. The genital vesicle of *Mya,* described above, seems to be less well defined in *Mercenaria* or *Spisula,* but these species do have greatly enlarged portions of the gonducts which occupy the same region.

The lining of the terminal gonoduct in *Mercenaria* is similar to that of *Mya.* On the other hand, that of *Spisula* is quite different. In this species the epithelium is a simple and relatively smooth layer of ciliated columnar cells, differing little from the ciliated epithelium on the exterior surface of the papillae (Fig. 5). Although some longitudinal folding is apparent, the prominent alternating ridges and furrows characteristic of *Mya* are absent.

Discussion

There are certain aspects of observed spawning processes in clams which appear to be related to the morphology of the reproductive system and which suggest the function of some of the structures observed in histological preparations. One of these aspects is the relative slowness of response to artificial spawning stimuli characteristic of *Mya.* Not only does this species respond much less readily than either *Mercenaria* or *Spisula* at any time, but its response declines noticeably toward the latter part of the spawning season. In 1961, when the laboratory spawning experiments were conducted, the period during which *Mya* could be found in a predominantly ripe condition lasted from late May through July, and some ripe individuals could be found even in mid-August. It was chiefly during the month of June, however, when this species responded most readily to spawning stimuli. Even during this brief period, the frequency at which spawning could be induced declined steadily during the period that any group of clams was held under observation. After the first week in July, no responses could be obtained, either from clams already under observation or from freshly dug specimens, even though many of them were still apparently ripe. On one occasion a group of clams expelled large quantities of blood and tissue cells, indicating a mechanical spawning reflex in response to the stimuli, but shed no gametes.

Examination of several hundred sections of *Mya* gonad tissue, representing all seasons of the year, has shown that rarely were ripe ova strongly preponderant in the ovaries. Usually, even in "ripe" individuals, eggs were present in all stages of development. It is difficult to understand, therefore, how a clam can emit several million eggs in a few minutes, as commonly occurs during spawning, if these must be freed directly from the ovaries.

What appears to be more likely is that eggs are accumulated before spawning in a reservoir from which they can be extruded *en masse*. This situation can be readily observed in both fresh and histologically prepared ripe specimens of both *Mercenaria* and *Spisula* (Fig. 5), where enormous numbers of ova (or spermatozoa) are accumulated in the enlarged portions of the gonoducts in the posterodorsal region of the visceral mass. It seems evident that the genital vesicle of *Mya* is homologous to the enlarged oviducts of the other two species and serves as a reservoir for the accumulation of gametes prior to spawning. By exerting pressure with a finger on the visceral mass of a ripe *Mya* in the locality of this vesicle, one can force from some individuals a limited quantity of gametes through the genital apertures. Once this quantity has been extruded, further pressure produces no more gametes, even though the ovaries are still replete.

If ripe gametes are accumulated in a reservoir prior to spawning, one might expect to find them in serial sections through the gonoducts and genital vesicle. Although only three of the serially sectioned specimens were ripe, one of these did contain substantial numbers of accumulated ova in the genital vesicle. Nevertheless, such accumulation was far less prevalent or exuberant in *Mya* than in either *Mercenaria* or *Spisula*. This fact could account for the relatively poor response of *Mya* to spawning stimuli if an accumulation of ripe gametes in a reservoir is a necessary precedent to spawning.

An extension of this argument may also help explain why apparently "ripe" clams in June spawn more readily in the laboratory than apparently "ripe" clams in July or August. Apparent ripeness is merely a measure of the abundance of ripe gametes in the gonad at a single moment. It indicates nothing about the rate at which they are being proliferated; yet the frequency of occurrence of clams with full reservoirs must certainly be dependent on the rate of gamete production. If the gamete production rate declines later in the season, response to spawning stimuli should decline accordingly.

Comparative studies of the three species have revealed another relationship between spawning physiology and morphology which, although its significance is not clear, warrants attention. In every experiment where *Mya* was induced to spawn in the laboratory, the eggs were shed with the nucleus membrane dissolved, that is, in the initial stages of meiosis. In this respect, *Mercenaria* is similar to *Mya*. On the other hand, *Spisula* releases eggs with the nucleus intact, and the initiation of meiosis ("activation") occurs after the sperm has penetrated the egg (Allen, 1953; Costello *et al.*, 1957). This is true of other lamellibranchs, and the American oyster (*Crassostrea virginica*), although not included among the species which I examined, is a pertinent example.

Apparently related to these differences is the fact that the eggs of *Spisula* and *Crassostrea* can be excised artificially from the ripe ovary and readily fertilized by placing them in clean water with a little sperm, after which they will develop normally (Costello, *et al.*, 1957; Galtsoff, 1937). Viable eggs can seldom be obtained in this manner from either *Mercenaria* (Loosanoff, 1953) or *Mya*. *Mya* and *Mercenaria,* the eggs of which are "activated" before spawning, and which cannot be fertilized successfully unless so "activated," both have the complex epithelium of the terminal gonoduct described earlier. *Spisula* and *Crassostrea,* which release their eggs before "activation," and from which viable eggs can be excised directly from the ovaries, have a relatively simple epithelium lining the gonoduct. This is shown for *Spisula* in Figure 7; Galtsoff (1961) shows a similar figure for *Crassostrea.*

Obviously, further investigation is required to clarify this relationship, and to determine the extent to which it applies to other species of lamellibranch.[1] Also, the examination of more individuals, collected in various stages of their seasonal reproductive cycle, should provide clues as to the role played by the epithelial cells in the terminal gonoduct.

SUMMARY

1. The reproductive organs of *Mya arenaria* consist of paired alveolar gonads, connected by paired gonoducts to a (usually) common vesicle. From the vesicle, a pair of short terminal gonoducts lead to the genital apertures.

2. The region occupied by the vesicle, terminal gonoducts and genital apertures is invested with muscular and connective tissue and is highly innervated with branchlets from the cerebro-visceral commissures which pass through the region. Paired branches from these nerve trunks also follow the main gonoducts into the gonad.

3. The terminal gonoducts are lined by longitudinally furrowed epithelium of ciliated columnar cells. In many individuals groups of these cells alternate with groups of non-ciliated, elongated cells with bulbous tips, apparently secretory in function.

4. Evidence is cited to show that sex products are accumulated in the genital vesicle prior to spawning. Homologous structures in two other species of lamellibranch are described which serve the same purpose.

5. An apparent relationship is shown between the time and site of egg activation and the nature of the epithelium of the terminal gonoduct of four species of lamellibranch molluscs.

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[1] Since the completion of this manuscript, I had occasion to examine sections through the genital papilla of the common mussel (*Mytilus edulis*) and found that the lining of the terminal gonoduct of this species is very similar to that of *Mya. Mytilus,* like *Mya,* also sheds its eggs after nuclear breakdown.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

1963

ABSTRACTS OF SEMINAR PAPERS

JULY 2, 1963

The scintillon—a new type of biological particle. RICHARD DESA, J. W. HASTINGS AND A. E. VATTER.

A new type of intracellular biological particle, which is functional in light emission, has been discovered and isolated. As seen with the electron microscope, it resembles a crystal. The particles, which we call "scintillons," occur in the photosynthetic marine dinoflagellate, *Gonyaulax polyedra.* The bioluminescence of this organism occurs as a short bright flash when mechanically agitated, and these particles evidently function in flashing. Scintillons are obtained by breaking cells and purifying by differential centrifugation. Their light-emitting potential remains relatively stable during purification; a luminescent flash (half-decay time, 0.08 second at 9° C.) is triggered specifically by lowering the pH to 5.7. The maximum initial intensity, as well as the number of photons emitted, is proportional to the number of particles over a 100,000-fold range of dilution. The time course of the flash is thus not dependent upon particle concentration. Under a polarizing microscope the scintillons can be shown to possess an extremely high degree of birefringence. Because of their small size and asymmetric shape, the scintillons exhibit a striking scintillation in polarized light. This is undoubtedly due to their rotatory Brownian motion. A similar scintillation, attributable to the oscillatory rotation of the particles, is readily visible in the living cell.

Inhibition of the bioluminescent oxidation of reduced flavin mononucleotide by 2-decenal. JAMES SPUDICH AND J. W. HASTINGS.

The bioluminescent reaction catalyzed by enzyme purified from luminous bacteria requires a long-chain fatty aldehyde for maximum light emission. It has been found that while the α,β unsaturated 10-carbon aldehyde (2-decenal) is completely inactive for light production, an aldehyde with the point of unsaturation more remote from the functional group is fully active. Moreover, the 2-decenal is a potent competitive inhibitor of the bioluminescent reaction. However, even under conditions where the reaction is strongly inhibited, the quantum yield of the reaction remains the same. The effect of this inhibitor in the bioluminescent reaction may be related to its stability against autoxidation. Whereas decenal is oxidized rapidly in aqueous solution, the 2-decenal is not. These experiments thus provide indirect evidence that the bioluminescent reaction involves the oxidation of the aldehyde to the corresponding acid.

Spermatozoan cholinesterase and coordination of the flagellar wave. LEONARD NELSON.

ATP induces spontaneous, repetitive, but discontinuous, flagellar twitching in glycerinated mammalian sperm. The inability of these "models" to move forward may be related to the loss of a mechanism which is instrumental in coordination and propagation of the flagellar wave. (However, reactivated sea urchin models reportedly can swim.) Glycerol removes some water-soluble constitutents, stabilizes some structural elements and apparently destroys the functional integrity of the plasma membrane. The failure of wave propagation has been attributed to the loss of cholinesterase (which has been found in boar and ram sperm and, in small amounts, in perch and trout sperm). In order to characterize the enzyme, washed bull epididymal sperm, fragmented by ultrasonic vibration (45 seconds at 20 kc.) was separated by differential centrifugation into head, midpiece and tail fractions. The cholinesterase activity of the tail fraction (125 μg. substrate hydrolyzed per milligram of protein per hour) was about 4 and 5 times greater than that of the midpiece and head fractions, respectively. Eserine (5×10^{-5} M) competitively inhibits the enzyme; the rate of hydrolysis of butyrylcholine is 15% and of benzoylcholine is about 5% of the rate of hydrolysis of acetylcholine. Moreover, the enzyme exhibits a pronounced substrate optimum relationship, being relatively inactive below a concentration 2.5 millimolar acetylcholine and strongly inhibited above 25 millimolar. The amount of hydrolysis increases with increased incubation time although the rate of hydrolysis is greatest after 10 minutes. These data indicate that the flagellar enzyme may be classified as a specific acetylcholinesterase. The fact that 5×10^{-5} M eserine increases the rate of flagellation of *Mytilus* sperm clearly suggests that acetylcholine and acetylcholinesterase may play a significant role in regulating the formation of the flagellar wave. The high enzyme content of bull sperm may reflect the need for a higher order of control in the morphologically more complex mammalian sperm than in fish and invertebrate sperm.

JULY 9, 1963

Heme-heme interaction in lamprey hemoglobin—an explanation. WARNER E. LOVE AND NEVENKA M. RUMEN.

Wald and Riggs, in 1953, and more recently Antonini and Wyman have found evidence of heme-heme interaction in the oxygenation reaction of lamprey hemoglobin. Since the work of Svedberg it has been thought that lamprey hemoglobin has but one heme group per molecule of molecular weight 18,000, in which case no possibility for heme-heme interaction can exist.

As a result of discussion with Dr. Riggs we studied the ultracentrifugal behavior of deoxygenated lamprey hemoglobin (*Petromyzon marinus*), and found that unfractionated hemoglobin associates to form tetramers when fully deoxygenated. Thus, the paradox of heme-heme interaction in a single-heme pigment is resolved, *i.e.,* the deoxygenated molecules combine with each other and the resulting polymer has more than one heme group per molecule, but oxy-, carbonmonoxy-, and met-hemoglobin are all monomers.

Lamprey hemoglobin is a microheterogeneous mixture of six molecules, all of the same molecular weight, which we have numbered 1 through 6 according to their electrophoretic mobility at pH 7.5. The individual hemoglobins show specificity in their combination upon deoxygenation. For example, hemoglobin 1 makes self-dimers, hemoglobin 4 makes self-tetramers but in an equimolar mixture only mixed dimers 1–4 are formed.

We speculate that these association reactions reflect steps in the evolution of modern vertebrate hemoglobins, in which there are two α and two β chains associated to form four-chain four-heme molecules.

This investigation was supported by research grant AM-02528 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.

JULY 16, 1963

A pressure analysis of the effects of heavy water (D_2O) on the form, movement and gel structure of Amoeba proteus. DOUGLAS MARSLAND.[1]

Specimens of *Amoeba proteus* immersed in deuterated Brandwein solutions, in which 30% to 98.8% of the H_2O content was replaced by D_2O, continued to sustain pseudopodial activity and locomotion for more than 8 days. At higher concentrations (90%–98.8% D_2O) the pseudopodia were unusually slender, elongate and tortuous, and frequently they took origin from the posterior extremity of the cell. The rate of streaming and locomotion was enhanced and the gel/sol ratio was definitely reduced by heavy deuteration.

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[1] Work supported by grant CA 00807, from the National Cancer Institute, National Institutes of Health.

Deuteration had a stabilizing effect upon the plasmagel structure of the pseudopodia. Higher pressures were required to solate the plasmagel to the point of instability, at which the pseudopodium behaves as a cylindrical mass of fluid and breaks into a number of separated beads. In 90% D_2O-Brandwein, it required 65,000 psi to evoke a definite beading of the pseudopodia, as compared with 4000 psi for specimens in H_2O-Brandwein solutions.

A stabilization of the plasmagel system was also evident when resistance to the sphering action of high pressure was plotted as a function of pressure, in media containing 30%, 70%, and 90% D_2O, respectively. The resistance/pressure curves were parallel to one another and to the gel strength/pressure curve. Each increment of D_2O concentration shifted the pressure values upwards by approximately 500 psi.

Double diploid DNA hypothesis in relation to mid-mitotic radioresistance of zygotes of Arbacia. RALPH HOLT CHENEY AND CARL CASKEY SPEIDEL.

Marked changes in DNA content occur from fertilization to first cleavage. A haploid amount of chromatin and DNA from the sperm nucleus combines with a similar amount in the egg. The early zygote thus contains a diploid amount. This amount *doubles* before first cleavage so that each daughter blastomere receives a diploid amount. Normal DNA replication and distribution via chromosomes at each cell division lead to normal development.

Gamma irradiation, which is penetrating, non-selective, and ionizing, alters DNA molecules and other chemical units. Ultraviolet (2537 Å) irradiation, which is less penetrating, very selective, and non-ionizing, also alters DNA, probably disturbing linkages within the molecule involving the thymine-adenine relationship. Radiation that alters DNA, the code-bearing material, leads to abnormal DNA replication and distribution at cell divisions and results in abnormalities in subsequent development.

We have found that *equal* exposures in a gamma dosage series (2–64 kr) to *Arbacia* zygotes at 5 successive stages of the first cleavage cycle (separate-pronuclei, monaster, streak, amphiaster, and cleavage-onset) induced *different* degrees of damage. Least damage and longest survival characterized embryos arising from zygotes irradiated at streak stage. *Streak,* therefore, was most radioresistant. Parallel experiments with UV (2537 Å) irradiation showed also that streak was most radioresistant.

We think that such radioresistance is correlated with DNA content; that DNA replication is essentially complete by mid-mitotic streak stage. This stage, then, contains a *double diploid* amount of DNA. It is, therefore, more radioresistant than earlier stages that have only a *single diploid* DNA content. Later stages, though still possessing double diploid DNA, become more radiosensitive because of the immediate influence of other factors associated with chromosome division and furrow formation.

We suggest also that the mid-mitotic stage of dividing cells in general may be more radioresistant than other stages of the cycle.

Supported by Grant GM 4326-07 to C.C.S. from the National Institutes of Health.

Motion pictures showing differential resistance of Arbacia zygotes to equal radiation dosages. CARL CASKEY SPEIDEL AND RALPH HOLT CHENEY.

Separate cultures of fertilized eggs of *Arbacia* were exposed to equal doses of gamma irradiation, 2–64 kr, at 16 successive times from fertilization to second cleavage. Illustrative cinephotomicrographs show the degree of injury in typical embryos that arose from zygotes given 12, 16, and 24 kr, respectively, at five developmental stages in the first cleavage cycle: (1) *separate pronuclei,* (2) *monaster,* (3) *streak,* (4) *amphiaster,* and (5) *cleavage-onset.* Stage 3 was most resistant, stages 1, 2, and 5 most vulnerable, and stage 4 transitional. A 1-unit gamma dose given at stages 1, 2, or 5 induced greater damage than a 2-unit dose at stage 3. The mid-mitotic phase of second cleavage corresponding to stage 3 (*streak*) of first cleavage was also relatively radioresistant.

Increase in radioresistance is ascribed to increase in DNA. Thus, the genetic *diploid* condition of stages 1 and 2 is succeeded by the *double diploid* condition of stage 3 *with respect to*

DNA amount. Replication of nuclear DNA molecules foreshadows the later visible chromosome division at stages 4 and 5. Each blastomere then receives a *single diploid* amount of DNA. By mid-mitotic phase of second cleavage DNA replication has again occurred, each blastomere then containing a *double diploid* amount of DNA. Double diploid is more radioresistant than single diploid. Vulnerability of stage 5, despite its double diploid DNA content, is ascribed to added radiation effects on other cellular constituents associated with proper furrow formation and chromosome distribution.

Similar experiments were done with 2537 Å ultraviolet irradiation, unilateral exposures, $\frac{1}{2}$–8 minutes. Stage 3 was again most resistant. Unique abnormal blastulae developed from many zygotes irradiated at stage 5 or later. At hatching time these consisted of a dead half attached to a live motile half. Autotomy occurred and the motile portion continued its development.

Supported by Grant GM 4326-06 to C.C.S. from the National Institutes of Health.

JULY 23, 1963

Polarized light microscopy by means of a birefringence detection system and scanning arrangement. ROBERT D. ALLEN.

A new method of polarized light microscopy (to be published fully by Allen, Brault and Moore in *Jour. Cell Biol.*) employs modulated light and narrow band detection in a photoelectric birefringence detection system (BDS) for scanning an object by means of light energy focused on a chosen specimen area by the condenser lens. Retardations due to birefringence within the selected area are registered as a voltage at a deflection sensitivity of 1 volt/Angstrom unit. The basal noise level for an integration time of 0.4 second and for a scanning area 36 microns in diameter in the oil immersion field is approximately equivalent to two milliangstroms of retardation. The noise level increases as the reciprocal of the diameter of the scanning area, but can be filtered with a consequent increase in the time required for a measurement.

The resolution is limited by classical Airy diffraction, and the speed and sensitivity are limited by the photon flux from the light source. As used in a conventional polarizing microscope, the BDS selectively registers birefringence. By the addition of suitable optical components, it can be made to register optical rotation, dichroism, and path difference due to refraction in interference microscopy.

This method is particularly useful in three situations frequently encountered in polarized light microscopy: (1) where extremely accurate measurements of retardations are desired at the highest resolution afforded by the optics, (2) where the object exhibits contrast due to properties other than birefringence (*e.g.*, light scattering), and (3) where changes in birefringence in a chosen portion of the specimen are to be followed with time.

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JULY 30, 1963

Limulus gill cartilage: a "plant-like" animal tissue. PHILIP PERSON AND DELBERT E. PHILPOTT.

In the light microscope, the histology and bio-architecture of *Limulus* gill cartilage are reminiscent of a parenchymatous plant tissue. In the electron microscope, structural details are evident, which are also similar to ultra-structural details of certain plant tissues. The following observations were offered in support of the above statements: (1) In both the light and electron microscopes, it may be seen that the matrix of the cartilage is very much like a plant cell wall. Regions of matrix are discerned, comparable in appearance with the middle lamella, primary and secondary cell walls of plants. (2) A process of cell-wall formation is seen in the cartilage tissue, using the electron microscope, which is remarkably similar in many respects to the process of phragmaplast and cell-wall formation in plants. (3) The cartilage tissue undergoes a process of accelerated chondrification, in which entire cells are chondrified and incorporated, *en masse*, into the matrix of the tissue. This phenomenon is very similar in appearance to a form of accelerated lignification which is seen in certain plants. Notwithstanding the differences in chemical composition between the gill cartilage and plant tissues, it is believed that the marked similarities between them, and between plant and cartilage tissues, generally, are not mere superficialities. These phenomena are deserving of more detailed investigation, now under way in our laboratories.

Analysis of tail regeneration in the lizard, Lygosoma laterale: the role of the ependyma. SIDNEY B. SIMPSON, JR.

Experiments were designed to test the importance of both the nerve contribution from the spinal cord and the ependymal lining of the central canal in the initiation of tail regeneration in the lizard, *Lygosoma laterale*. Results from these experiments demonstrated that in the presence of the ependymal lining of the central canal, tail regeneration could occur after removal of as many as three-quarters of the nerves contributed by the spinal cord. Results were also obtained that demonstrated the ability of the ependymal lining of the central canal to induce tail regeneration. These results were interpreted to mean that the ependymal lining of the central canal is the primary initiator of tail regeneration in this lizard.

Experiments were also designed to test the importance of the ependyma in the induction of cartilage. Results from these experiments demonstrated that: (1) the absence of the ependyma in a regenerate was always associated with the absence of a cartilage tube; and (2) autografts of ependyma transplanted to normal autotomized tails resulted in the induction of accessory cartilage tubes. These results were interpreted to mean that the ependymal lining of the central canal and/or its associated glial cell component is responsible for the induction of cartilage in the lizard tail.

Ebb-tide antigens in the brain. MAURICE M. RAPPORT.

Despite the fact that crude lipids have long been known to react with antibodies, the chemical relationship of lipid structure to immunological activity has heretofore been poorly defined. Four classical problems in this area were the chemical nature of the Wassermann antigen, the structure of Forssman hapten, the organ specific hapten in brain, and the substance responsible for the large differences observed between the serological activity of lipid extracts from neoplastic and from normal tissues. With human tissues, a partial solution to this last problem was provided by the isolation and identification of cytolipin H, a molecule with four residues, one each of fatty acid and sphingosine, and one each of glucose and galactose in the lactose configuration. Studies with pure cytolipin H showed that glycosphingolipids of relatively simple structure could function as haptens (partial antigens), but that rather large amounts of auxiliary lipids (lecithin plus cholesterol) were required to detect activity. The existence of a lipid hapten present in the brains of all mammals but not in other tissues was first reported in 1926 and repeatedly confirmed. Attempts to identify the reactive substances were unsuccessful, and although in 1934 it was reported to be associated with the glycosphingolipid fraction, its identity with galactocerebroside (the major lipid constituent of brain, discovered by Thudichum 80 years ago) was firmly denied. Within the past year, we established that these reports were incorrect and that galactocerebroside is indeed responsible for the brain specificity (Nature, 1963: *197,* 60). The earlier error, reported during the flood-tide of scientific interest in carbohydrate residues as antigenic determinants under such powerful leaders as Landsteiner, Avery, Goebel, Heidelberger, Marrack, and Harington, permanently stunted development of interest in "lipid" haptens. The ready availability of galactocerebrosides and their molecular simplicity in containing only one carbohydrate residue would have provided a superb bridge from the artificial antigens so vigorously studied to those which occur in animal cells.

AUGUST 6, 1963

A thermodynamic definition of hydration. MAX A. LAUFFER.

Newton and Gortner and also Hill defined "bound" water as water not free to dissolve added solutes, as determined by freezing point or vapor pressure depression, respectively. Because of inherent thermodynamic ambiguities, a simpler definition was sought. If anhydrous uncharged colloid is added to a binary solution (glycerol in water), the mole fractions of solvent and of solute will decrease by equal fractions; the depressions of logarithms of mole fractions of solvent and solute will be identical. Because the colloid usually affects differently the escaping tendencies of solvent and solute, the depressions of logarithms of activities of solvent and solute will differ.

Define as "hydrate" that amount of solvent which when added simultaneously with the anhydrous colloid produces identical depressions of logarithms of activities of solvent and solute. This is equivalent to adding hydrated colloid. Now the depressions of the logarithms of activities, of mole fractions, and, therefore, of activity coefficients are, respectively, identical for "free" solvent and solute on the "free" basis. Furthermore, one can prove from the Gibbs-Duhem relationship that, provided only that colloid is osmotically ideal, the activity coefficients of "free" solvent and of solute on the "free" basis are identically equal to those of solvent and solute before addition of hydrated colloid, and, even for a very concentrated (mole fraction, one ten thousandth) very non-ideal (osmotic coefficient, four) protein solution, they differ by only three parts in ten thousand.

These conclusions are valid for protein salts dissolved in multicomponent solutions including electrolytes when "binding" of other constituents is accounted for. This definition of hydration, differing quantitatively only slightly from its predecessor and retaining the dependence on solvent activity (Briggs), thus has the advantage of leading to simple thermodynamic relationships among the "free" constituents.

Membrane equilibrium in multicomponent systems. MAX A. LAUFFER.

In 1937 Adair, starting with the Gibbs relationship between chemical potential, pressure and activity at constant temperature, which must be obeyed by all permeable constituents, derived the relationship at osmotic equilibrium between the activities of the solvent and of the permeable solute on the two sides of an ideally semipermeable membrane separating a solution containing nonpermeable colloid from one without colloid. In this case, the change in logarithm across the membrane of the activity of the solvent is equal to that of the permeable solute plus a term involving the effect of the pressure difference at osmotic equilibrium on the partial molal volumes of solvent and permeable solute. A small amount of the constituent with higher molal volume will be squeezed out of the colloid solution because of the higher pressure. Equations were derived both for systems containing non-electrolyte and uncharged colloid and for systems containing electrolyte and charged colloid. The latter represents an exact treatment of Donnan equilibrium. To extend this analysis to mole fractions and molalities, it is necessary to know the activity coefficients of permeable solute and of solvent on both sides of the membrane. Adair did not solve this problem.

We found the same relationships to apply to all permeable components of multicomponent systems. As shown above, activity coefficients on the "free" basis of all permeable components are identical with those in a solution of the same composition except for colloid and "bound" material. Therefore, the composition of "free" components in a protein solution at osmotic equilibrium can be determined unambiguously. For the system, protein, water and solute, "hydration" can be measured by weighing at equilibrium the protein solution in an ideally semipermeable sac suspended in water and solute.

Electron miscroscope studies of chromatoid bodies of axenically-grown trophozoites of Entamoeba invadens. WASIM A. SIDDIQUI AND MARIA A. RUDZINSKA.

There have been conflicting reports in the literature about the chemical nature and origin of chromatoid and related bodies found in the cytoplasm of certain protozoa and in the spermatogenic cells of many metazoans. Recently Barker has shown in his cytochemical studies that the chromatoid bodies in *Entamoeba invadens,* a parasite of snakes, are composed of ribonucleoprotein. The object of this paper is two-fold: (a) to show that the chromatoid bodies, which are characteristic of precyst and cyst stages only, are also found in axenically-grown trophozoites, where there is no cyst formation, and (b) that the ribonucleoproteins within the chromatoid bodies are arranged in a helical pattern.

Axenically-grown trophozoites of the PZ strain of *E. invadens* were fixed in 1% OsO_4 buffered with veronal acetate at pH 7.4 and embedded in cross-linked methacrylate. Thin sections were examined with an electron microscope. In the cytoplasm of a single trophozoite may be seen 2–6 chromatoid bodies, each composed of 9–34 lamellae in parallel arrays. The lamellae are 250 to 350 Å wide and 100 to 150 Å apart. At higher magnification, they appear to be composed of coiled fibrils forming a closely packed helix.

This is the first demonstration by electron microscopy of a helical structure in ribonucleoprotein bodies in protozoa. Since the structure disclosed seems important enough to be reflected in terminology, and since the terms previously used have often been confusing, it seems appropriate to introduce a new, more specific term, "helical RNP bodies," for chromatoid bodies of the same nature as those found in *E. invadens*. The new term combines the biochemical composition defined by Barker with the helical structure disclosed in this paper.

Effect of blood pressure on the isolated tunicate heart. MAHLON E. KRIEBEL.

The direction of the contraction wave is periodically reversed every ten to 40 minutes, due to alternating activity of two pacemakers located at each end of the heart. Reversal of heartbeat was originally explained by a change in blood pressure which altered the excitability of the pacemakers. Recently it has been suggested that a "periodic fatigue" of a pacemaker occurs, since isolated hearts may reverse and half-heart preparations may show periods of activity and rest. The effects of (1) varying the amount of blood, (2) changing the internal blood pressure, and (3) increasing the peripheral resistance in an artificial circulatory system were measured on the isolated *Ciona intestinalis* heart. The 15-mm.-long V-shaped hearts were supported by the pericardium which was held in a clamp. A strain gauge was attached to each end of the heart and the wave of contraction was recorded on a Grass polygraph. (1) Completely deflated hearts showed no periodicity and contracted irregularly at 3 to 10 beats/minute. Contractions in half-full hearts reversed ten times (1–4 minutes) more often than when normally full. Further distention lengthened and finally abolished the reversal period. One side generally became dominant in distended hearts and rhythmically paced the heart at 20 to 40 beats/minute for several hours without "fatiguing." (2) Internal pressure was varied by altering the height of reservoirs connected to cannulas inserted into each heart vessel. An increase of 2 mm. water pressure increased the beat frequency from 9 to 22, 22 to 52 and 7 to 33 contractions/minute in three hearts. After a normal reversal, the heart could be reversed again by increasing the blood pressure by 2 mm. in the side which had just stopped. (3) Isolated hearts which were allowed to pump blood in a closed circuit did not reverse for one hour. But when the circuit was closed, reversal occurred within four beats. This is evidence that blood pressure affects the excitability of the pacemakers and influences the periodicity of reversal in the isolated heart.

AUGUST 13, 1963

Lysosomes in thyroid epithelium of untreated, TSH-stimulated and I^{131}-irradiated rats. ALEX B. NOVIKOFF.

With Dr. A. Vorbrodt we have studied thyroids from the three groups of rats by: (1) electron microscopy of thin sections of Epon-embedded material; (2) light microscopy of frozen sections stained for acid phosphatase, other phosphatase and esterase activities; and of paraffin and Epon sections stained by the PAS technique; and (3) electron microscopy of thin sections of frozen sections incubated for enzyme activities. Cells of untreated rats possess "dense bodies" with hydrolase activities; a variable number of cells have larger "droplets" with such enzyme activities. Following TSH stimulation most cells show such droplets; these are identical with the PAS-positive "colloid droplets." The evidence is tentatively interpreted in the following manner. When the droplets form at the cell surface (pinocytosis) they receive acid hydrolases by fusion with dense bodies ("pure" lysosomes?). The absorbed colloid is hydrolyzed within the pinocytosis vacuoles ("derivative" lysosomes) and thyroid hormone is liberated and transported into adjacent capillaries (whose endothelial cells have high nucleoside phosphatase activity in numerous micropinocytosis vacuoles). Between the first and third day following injection of a high dose of I^{131} all signs of secretory activities disappear. The endoplasmic reticulum fragments and becomes largely denuded of its ribosomes. The Golgi saccules flatten. Golgi-associated vacuoles (perhaps carrying synthesized material to and from the saccules) are no longer present. Colloid droplets and small apical vacuoles also disappear. The Golgi "vesicles" ("pure" lysosomes?) remain and the dense bodies are numerous. Lysosomes are among the last structures to decrease in injured cells. Late in cell injury, much enlarged lysosomes contain materials probably resulting from digestion of cell constituents. Thus, under some circumstances lysosomes can be "suicide sacs" (de Duve). Under normal conditions they may play important roles in organismic physiology as well as cell physiology.

Nucleo-cytoplasmic interactions in the development of the salivary glands of Chironomus thummi (Diptera). HANS LAUFER, YASUKIYO NAKASE AND JEROME VANDERBERG.

The polytene chromosomes of *Chironomus thummi* larval salivary glands have puffs, regions of less densely coiled chromosomal fibers that are centers of heightened RNA synthesis. Enhancement and repression of puffs occurs in particular patterns in the course of development, which are characteristic for particular tissues. Hormones control the development of the puffs, the organism, as well as the salivary glands, which double in size during the fourth instar, only to degenerate during the larval-pupal transformation. These observations support the concept that development is an orderly sequence of gene activations and inactivations, in which hormones act directly or indirectly as regulators of genes. It also follows that the largest puffs should be concerned with the major functions of the gland.

We sought correlations between specific cytoplasmic activities and three most active puffing loci (nucleolus and the two Balbiani rings at region *b* and *c*). Seven enzymatic activities were followed during glandular growth and destruction: hyaluronidase, trehalase, and protease were found in the secretion; malate dehydrogenase, esterase, ribonuclease, and deoxyribonuclease were restricted to the gland. All other enzymes except the nucleases increased significantly in activity; some more than doubled during the fourth instar, only to decrease at metamorphosis, following the pattern of glandular growth and protein accumulation. These changes in enzyme activities are paralleled by changes in the activities of the nucleolus and the Balbiani rings, which first expand and then regress before pupation. RNAse remains constant throughout but DNAse activity is detected only at the end of larval life, when the gland breaks down. DNAse activity correlates best with what Clever has called developmentally significant puffs. The increase in DNAse is sufficient to account for the glandular destruction during development.

Wounding, hormone deprivation, and treatments (actinomycin D and puromycin) interfere with normal development. They also affect puffing of the Balbiani rings, the nucleolus, and enzymes in characteristic ways, supporting the view that development involves differential gene activation.

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AUGUST 20, 1963

Dynamics and energetics of the circulatory system of dog fish. FREDERICK N. SUDAK.

Intracardiac, aortic, and intrapericardial pressures were measured in order to determine the magnitude and interrelationships of pressures in the cardiovascular system of *M. canis* and *S. acanthias*. Animals were immobilized by means of a mid-diencephalic section and destruction of the spinal cord posterior to the twentieth vertebra. Pressure measurements were obtained directly from the heart chambers, pericardium, and ventral aorta by means of 18-gauge needles or 0.044″ catheters attached to pressure transducers. Atrial systolic pressure ranged from 1 mm. Hg to 5 mm. Hg. Pressure within this chamber averaged 2.2 mm. Hg below atmospheric pressure during early diastole. Ventricular systolic pressure ranged from 20 mm. Hg to 50 mm. Hg. Early diastolic pressure averaged 1.6 mm. Hg below atmospheric pressure in this chamber of the heart. End-diastolic pressure in the atrium and ventricle was 1.3 mm. Hg below atmospheric pressure and 0.6 mm. Hg below atmospheric pressure, respectively. Intrapericardial pressure ranged from 1 mm. Hg to 7 mm. Hg below atmospheric pressure. Intrapericardial pressure affected pressures developed in the heart chambers, rate of intracardiac pressure change during diastole, and end-diastolic pressures. When intrapericardial pressure was made equal to atmospheric pressure, pressure developed by the ventricle was significantly reduced. Ventricular and atrial pressures remained above atmospheric pressure during diastole. Rate of pressure change during diastole was significantly prolonged in both chambers of the heart.

These data confirm earlier reports of "negative" pressures in the venous system of elasmobranchs. Furthermore, the pericardium of these animals plays a significant role in cardiac function. Evidence is presented to suggest a functional role of the pericardium in facilitating venous return to the heart.

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The pigmentary system of the squid (Loligo pealii). GEORGE SZABÓ, THOMAS B. FITZPATRICK AND GEORGE WILGRAM.

The pigmentary system of the eye, skin, and the ink gland was investigated. In all three loci it is associated with the light-reflecting layer of iridiocytes. The pigment is granular in all three locations. It is ommochrome in the eye and in the skin, and melanin in the ink gland. Electron-microscopic investigation revealed that the ommochome granules of the eye and chromatophores of the skin are about the same size or larger than mammalian melanin granules (0.1 micron) and have internal striation when not fully pigmented. The smooth muscle cells around the chromatophores of the skin have a very large nucleus and bands of muscle fibers run at acute angles to the cell membrane of the chromatophore.

The epithelial cells of the ink gland are true secretory cells, with extensive basal infoldings towards the lumen of "capillaries." They are polarized, with a highly developed rough-surfaced endoplasmic reticulum towards the cell base and a vacuolated cytoplasm towards the apical pole. Several melanin granules are located in vacuoles at the apical pole. The granules are very small, about half the size of mammalian melanosomes (0.05 micron or smaller). They are a fraction of the size of the very large mitochondrion (0.1–0.2 micron in many cases). The melanin granules are secreted into the lumen of the gland, where they are not aggregated. The ink cells bear several cilia.

The iridiocytes are similar in all three locations (eye, skin, ink sac). They contain an intricate system of parallel lamellae in the newly hatched squid. These lamellae are well preserved in electron microscopical preparations—they cannot be guanin crystals, as previously assumed.

The biochemical analysis of the pigment of the eye and the ink gland showed the absence of succinoxidase in the ommochrome pellet of the eye and in the melanin pellet of the ink or ink gland. Tyrosinase was present in the ink and it was also traced in the ommochrome. Characterization of tyrosinase is under investigation.

Stimulation by specific quinones of glucose metabolism by brain. F. C. G. HOSKIN AND CAROLE VON ESCHEN.

Mammalian brain normally produces $C^{14}O_2$ in equal amounts from either glucose-1-C^{14} or G-6-C^{14}, indicating, for brain, glucose metabolism virtually exclusively *via* the Embden-Meyerhof pathway. At about 10^{-5} *M*, menadione, plumbagin, trimethylquinone, 2,3-dimethyl-1,4-naphthoquinone, 2-methyl-5,6,7,8-tetrahydro-1,4-naphthoquinone and 2-methyl-4a,5,8,8a-tetrahydro-1,4-naphthoquinone cause a 2- to 3-fold increase in $C^{14}O_2$ production from G-1-C^{14} but are without effect on G-6-C^{14}. Up to 5×10^{-5} *M*, quinone, toluquinone, m- and p-xyloquinone, 1,4-naphthoquinone, 5,8-dihydroxy-1,4-naphthoquinone, phthiocol and droserone are without effect on either G-1-C^{14} or G-6-C^{14} metabolism. At higher concentrations they become generally inhibitory. Juglone and lawsone show some stimulation at 1 or 2×10^{-5} *M*, marked inhibition at higher concentrations. Vitamin K_1 and coenzyme Q_{10} show slight general stimulation, *i.e.*, of both G-1-C^{14} and G-6-C^{14} metabolism to $C^{14}O_2$.

Turning now to a different test reaction, menadione, plumbagin and 2,3-dimethyl-1,4-

naphthoquinone are highly active in catalytic amounts in stimulating G-6-P metabolism and TPNH oxidation by soluble guinea-pig preparations. Quinone, toluquinone, m- and p-xyloquinone, trimethylquinone, lawsone, phthiocol, droserone, and the two tetrahydro members are either completely inactive or active only in stoichiometric, rather than catalytic, amounts. Juglone, 1,4-naphthoquinone and 5,8-dihydroxy-1,4-naphthoquinone have some catalytic activity. Vitamin K_1 and coenzyme Q_{10} show anomalous behavior which may be due to problems of solubility. The menadione stimulation is inhibited by an equivalent concentration of dicoumarol but not by the inactive members, *e.g.*, phthiocol. The two tetrahydro members, despite their inactivity in the soluble preparation, continue to stimulate $C^{14}O_2$ production from G-1-C^{14} by brain slices. These results indicate an interaction of specific quinones with specific enzymes—enzymes whose distribution in soluble fractions does not accurately reflect their distribution in intact tissue.

Flagellar regeneration: a mechanism accounting for its initiation and regulation.

FRANK M. CHILD.

We have demonstrated that the kinetics of flagellar regeneration in *Chlamydomonas* and *Astasia* are the same as those discovered by Dubnau (1961, Ph.D. Thesis, Columbia) in *Ochromonas;* namely, a lag period involving no elongation followed by a period of elongation showing a gradually diminishing rate. In studies of these kinetics in single cells of a paralyzed mutant of *Chlamydomonas,* which have had their two flagella unequally amputated, preliminary results show that the lag is dependent on stump length; *i.e.*, shorter stumps have shorter lags; but the rates of elongation differ so that both stumps appear to culminate simultaneously at the same final length.

These results indicate that part of the control and regulation of regeneration resides within the shaft of the flagellum itself. I suggest that this control involves the proximo-distal gradient of connectives anchoring the membrane to the axoneme. This gradient is demonstrated by the osmotically controllable pattern of membrane-ballooning induced under certain conditions. The mechanism proposes that: this gradient is a gradient of macromolecular organization induced and maintained by the terminal dominance of the tip; the stronger connectives at the base of the mature flagellum have properties which resist the insertion of more macromolecules into the growth zone, assumed to be located at the base of the shaft; during the lag period, the gradient of the stump is reorganized by its new tip; at the base of the reorganized stump, the reduced strength of the connectives allows elongation at a rate inversely proportional to that strength. This mechanism would explain the feed-back kinetics of elongation, the nature of the lag period, and certain other phenomena involving variations of flagellar length. It appears to be testable.

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AUGUST 23, 1963

Sperm morphology of Emerita talpoida.

K. R. BARKER AND C. R. AUSTIN.

Spermatozoa of *Emerita talpoida,* the mole crab, are non-flagellated as is typical of many crustaceans. Observations showed that they consist of a "head" which contains the nuclear material, a number of filaments, a "body" and an acrosome at the distal end of the body. From three to nine filaments, spaced equidistantly, were seen in different spermatozoa; they were often stuck together when first recovered and sometimes could be seen separating. In spermatozoa treated with hypotonic solutions, the filaments collapsed into small rounded masses on the surface of the head. Unlike crayfish spermatozoa (Moses, 1961), those of *E. talpoida* contained no nuclear material in the filaments which were negative to Feulgen and acridine-orange staining. When spermatozoa were stained with acridine orange solution and viewed with a UV light source, the head fluoresced green and the acrosome a faint red. Some spermatozoa spontaneously underwent the acrosomal reaction. In these cases, green fluorescent (and Feulgen-positive) material was observed also in the body. This would seem to indicate that when the acrosome reaction occurs, nuclear material passes into the body. By electron micrography, a tube, which in cross-section was seen to be subdivided into four parts, was found to occupy the center of the body. Spermatozoa of *E. talpoida* showed no motility at any time, even when brought in contact with body-cavity eggs or fertilized eggs. A structure resembling a mitochondrion was observed around the body just below the nucleus.

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Fine structure of Nereis limbata spermatozoa. J. F. FALLON AND C. R. AUSTIN.

Spermatozoa of *Nereis limbata* were prepared for electron microscopy by osmium fixation immediately after shedding. The spermatozoon has four delimited areas: acrosome, nucleus, mitochondrion and flagellum. The cell is surrounded by a plasma membrane. The acrosome membrane lies directly beneath the plasma membrane except for the portion adjacent to the nucleus. At the midpoint of this region, the acrosomal membrane invaginates to form an oblong chamber extending more than three-quarters the length of the acrosome and ending in a small, round vesicle. The chamber houses the anterior portion of the rod-like perforatorium, which is one-third the width of the chamber and extends, slightly coiled, to the terminal vesicle. The posterior portion of the rod lies in an invagination of the nucleus lined by nuclear membrane. The acrosome contains a hollow cone of dense granular material which surrounds the rod chamber. The tip of the acrosome contains an apical cavity with extensions running the length of the acrosome and surrounding the hollow cone. The apical cavity and its extensions are bounded by a less granular material which lies subjacent to the acrosomal membrane. The anterior part of the nucleus contains indentations extending inwards from the anterior surface, which are filled with material less electron-dense than the nuclear substance. A single large mitochondrion is disposed around the posterior pole of the nucleus, where it surrounds the beginning of the tail. The base of the tail makes an indentation into the posterior portion of the nucleus. A cross-section through this region shows the centriole as a circle of electron-dense material with a light core, and nine non-radial projections with expanded ends extending from the outer part of the circle.

Grateful acknowledgment is made to Fisher Scientific Co. and L.K.B. Instrument Co. for loan of a JEM-T6 electron microscope and an Ultrotome, respectively. Aided by Training Grant 5T1GM988-02 from the National Institutes of Health.

Penetration of oil drops into Echinarachnius eggs. N. L. GERSHFELD.

Some of the oil penetration experiments of Kopac and Chambers were repeated and their findings verified, using sand dollar eggs, whose jelly coats were removed in pH 3.5 sea water, and a purified grade of mineral oil.

The following model is proposed to describe the process of oil penetration: the jelly-free egg is covered by a membrane which exhibits fluid properties, and (a) upon contact of the oil drop and egg, a portion of the egg membrane flows over the oil drop as a film, creating a metastable system in which the interfacial tension is uniform over both the oil drop and the egg; (b) a stable state arises when the surface/volume ratio is a minimum; this occurs when the oil drop is enclosed within the egg; (c) the membrane area disrupted by the oil drop reforms and seals. Step (a) is a very rapid process (rates of monolayer spreading on liquid surfaces can be as high as 10 cm./sec.), as is step (b); step (c) is relatively slow, of the order of several minutes.

This model permits the following analysis: the conditions under which the egg film will flow over the oil drop are determined by the Harkins spreading coefficient: $S = \gamma$(oil/water)$-\gamma$(egg/water)$-\gamma$(egg/oil), where γ is the interfacial tension of the indicated interface. When $S > O$ the membrane-film will flow over the oil drop; but when $S < O$, oil drop penetration will not occur.

It is possible to control the spreading process by reducing γ oil/water by the adsorption of surface-active material from the water phase into the interface, and this is demonstrated by the following: If an oil drop is formed and maintained in sea water for varying periods of time (10–30 seconds) γ(oil/water) decreases continuously, as indicated by a pronounced Devaux effect. One finds complete penetration, partial penetration, or no penetration of the oil drop into the egg with increasing time of exposure to and soluble organic content of the sea water. However, in artificial M. B. L. sea water complete penetration by oil drops is observed even after prolonged exposure to sea water (5 minutes). These results support the proposed model.

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Purification and characterization of Mytilus egg membrane lysin from sperm. STEPHEN D. HAUSCHKA.

An egg membrane lysin from sperm (Berg, 1950), responsible for destroying the hyaline layer (Dan, 1962), has been purified, and some parameters of the lytic process studied. In a typical purification, 250 grams (wet) testes were minced and stirred into 500 ml. Tris buffer (pH 8.2, ionic strength 0.5 with NaCl). The sperm suspension was centrifuged 10 minutes at 20,000 *g;* the pellet was resuspended in 150 ml. buffer, and freeze-thawed to release the lysin. The extract was centrifuged 45 minutes at 30,000 *g,* and the supernatant recentrifuged one hour at 78,000 *g.* The high-speed supernatant was adjusted to 20% saturation (0° C.) with solid $(NH_4)_2SO_4$ and stored ten hours at 0° C. The 20% precipitate was removed by centrifugation, and the active fraction was then precipitated by increasing the supernatant salt concentration to 30%. After centrifugation the precipitate was suspended in 10 ml. buffer and dialysed overnight against the buffer. This preparation contained a single peak sedimenting at 3S and a minor shoulder (5%) sedimenting slightly faster.

Lytic activity was assayed by timing the disappearance of the hyaline layer from eggs suspended in sea water. Dilutions of the lysin exhibited linear rate dependence upon concentration. The lysin was inactivated by heating one minute at 100° C., and was non-dialysable. Trypsin duplicated the lysin's effect upon the hyaline layer; but the lysin showed no proteolytic action upon serum albumin.

Antisera prepared against freeze-thaw extracts of sperm and eggs were tested upon the assay system. Anti-sperm produced no effect upon the lytic rate, and no precipitin band was formed against the purest lysin sample. Anti-egg, likewise, produced no effect when mixed with the lysin. However, eggs pre-incubated five hours with anti-egg serum became completely resistant to lytic attack, while eggs pre-treated with anti-sperm or non-immune serum exhibited no such behavior.

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Studies on the soluble antigens of the sperm of Arbacia punctulata. JOHN A. KLOETZEL AND C. B. METZ.

The extractable material of *Arbacia* sperm, prepared by freeze-thawing and 10,000 × *g* centrifugation, includes at least four antigens. These form four precipitin bands when diffused in agar against anti-whole *Arbacia* sperm serum, and are designated a, b, c, and d, beginning at the antigen well. This study was undertaken to find which of these antigens is involved in the fertilization reaction.

Such an antigen is present in freeze-thaw extracts, since absorption of anti-whole sperm serum with excess extract neutralizes its fertilization-inhibiting action (Bischoff and Metz, 1962). This antiserum-neutralizing factor is apparently sedimented by 30,000 × *g* centrifugation. Flake and Metz (1962) showed that absorption of antiserum with excess whole sperm also neutralized its fertilization-inhibitory action, yet left two band-forming antibodies in the serum.

In the present study, anti-*Arbacia* sperm serum, following absorption with an equal volume of packed sperm given in three doses, produced only the a and c bands when diffused against sperm extract. In cases where unabsorbed antiserum produced only three bands, the corresponding absorbed serum produced the a band alone. Centrifugation of an extract at 40,000 ×*g* in four out of six cases left a supernatant which failed to produce the b band when diffused against unabsorbed antiserum. When resuspended, the 40,000 × *g* precipitate produced only the b band (two experiments). In two other cases, additional bands were found with this material, indicating contamination with the supernatant.

Comparison of these results with those of earlier workers indicates that the antigen(s) producing the b band is the one of importance in fertilization. Previous studies indicate (Metz and Köhler, 1962) that antigen b is restricted to the sperm head, whereas antigen d, the other surface antigen (Flake and Metz, 1962), is found in extracts of both isolated heads and tails.

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Ultrastructure of Pectinaria gouldii gametes. Roger Lambson and C. R. Austin.

The procedure recommended for tissue embedding with Cargille araldite has been modified and yields suitable blocks for ultra-thin sectioning if exact quantities of the constituents are used and moisture is rigidly excluded. Infiltration and polymerization require less than two days.

Gamete suspensions from male *Pectinaria gouldii* contained gametes in all stages of maturity. A graded series of bundles was recovered, showing an inverse proportion between cell number and cell size as maturity was approached. Electron microscopy showed that the common body of cytoplasm attaching spermatogenic cells to one another contained few organelles, as Calkins (1895) had implied for similar structures in *Lumbricus*. Large mitochondria were disposed around each nucleus, and endoplasmic reticulum, often found as vesicles, was scattered in the cytoplasm. A prominent Golgi apparatus was noted in many cells adjacent to the nucleus on the side toward the attachment.

Gametes from female worms showed clumps of several large cells. However, in the coelomic cavity, the maturing eggs evidently break away from these masses while quite small, and mature as isolated cells. The prominent microvilli increased in length during germinal vesicle breakdown after shedding and the eggs rounded up with an apparent decrease in diameter.

Intact germinal vesicles showed a very irregular outline, and small electron-dense bodies were found in a layer outside the nuclear membrane, while a few were scattered in the cytoplasm along with the mitochondria and some homogeneous vesicles. The plasma membrane had an irregular outline and microvilli protruded through the rather amorphous vitelline membrane.

Grateful acknowledgment is made to Fisher Scientific Co. and L.K.B. Instrument Co. for loan of a JEM-T6 electron microscope and an Ultrotome, respectively. Aided by Training Grant 5T1GM998-02 from the National Institutes of Health.

An electron micrograph study of the action of cysteine on the cell surface of frog eggs. Arlan E. S. Smith.

Fertilized and unfertilized eggs of *Rana pipiens* were treated with 0.1 *M* cysteine in 1/5 Holtfreter's solution, pH 7.8, approximately 30 minutes after fertilization or stripping. After dissolution of the jelly coat by cysteine and thorough washing, the eggs were fixed for 3 to 5 hours in 1% osmium veronal-acetate buffer and embedded in Araldite 6005. Sections were stained in uranyl acetate and viewed in the JEM-T6 electron microscope.

The cell surface of fertilized eggs treated with cysteine was highly convoluted, possessed many large projections, and almost totally lacked an electron-dense surface membrane. The surface was mainly composed of relatively homogeneous small granules. Electron micrographs of unfertilized cysteine-treated eggs revealed that the surface membrane remained relatively electron-dense, that numerous large and small microvilli projected from the surface, and that cortical granules were not broken down. Cortical granules appeared to accumulate and pack at the surface. In cysteine-treated, artificially activated (pricked with a fine glass needle) unfertilized eggs, it was found that cortical granules broke down and that a uniform relatively electron-dense surface membrane formed.

The disorganization of the egg surface in cysteine-treated, fertilized eggs indicates that disulfide bonds are of importance for the maintenance of the integrity of the electron-dense surface membrane of the frog egg. The presence of numerous microvilli and the fact that the vitelline membrane lifts from cysteine-treated, unfertilized eggs suggest that disulfide bonds serve as links between the surface of the microvilli and the vitelline membrane. Cortical granules closely resemble lysosomes in size, electron-density, and granularity, suggesting that they may possess lysosome-like activity which may be related to the breakdown and release of cortical granules during fertilization.

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Cross reaction between sperm and fertilizin of Arbacia from Florida and Woods Hole. SAMUEL STERN AND C. A. SHIVERS.

The opportunity presented itself for us to compare some properties of fertilizin obtained from eggs of *Arbacia* from Woods Hole (WF) and Panama City, Florida (PF). Presently, the animals from both regions are classified as *A. punctulata.* Morphological differences are observed between animals from these two regions. For example, the color of the southern urchin is usually a uniform dark purple, whereas the northern *Arbacia* (Harvey, 1956) is much more variable in color. The average spine length of animals, which varied between 3 and 4 cm. in body diameter, was 2.62 cm. for the southern form and 1.40 cm. for the northern animal.

Acid-extracted fertilizin, neutralized by dialysis against sea water, was obtained from both forms of urchins. These fertilizins were serially diluted and titered with a 2% suspension of sperm from both the Woods Hole form (WS) and the Florida urchin (PS). In five experiments we found that at all times WF agglutinated WS better than did PF; PF in general agglutinated PS better than WS, but PS was equally well agglutinated by PF and WF.

Fertilizins were added to 2% sperm suspensions, in all possible combinations, until the sperm no longer agglutinated. After reversal of agglutination the sperm were mixed in all possible combinations with equal volumes of fertilizins. For example, PS was agglutinated with WF and PF and after reversal was mixed with WF and PF. The only combination which resulted in further sperm agglutination was WS agglutinated with PF and then mixed with WF.

These data suggest that there are at least two reactive sites on the sperm and/or fertilizins: those specific for the homologous form and those common to both forms.

Further studies are in progress to determine the location and chemical nature of the sites.

This project has been aided by Training Grant 5T1GM998-02 from the National Institutes of Health.

Inhibition of fertilization by specific antibodies dissociated from sperm. D. E. WILLIAMS AND C. B. METZ.

If *Arbacia* sperm are treated with univalent antibody prepared against whole *Arbacia* sperm, the fertilizing capacity of the sperm is reduced (Metz and Schuel, 1961). This effect upon fertilization cannot be removed by adsorbing the antiserum with the cross agglutinating sperm of *Echinarachnius.* The preceding suggests that *Arbacia* antigens shared with *Echinarachnius* do not function in fertilization. However, it is possible that the antibodies adsorbed out by *Echinarachnius* sperm could reduce the fertilizing capacity of *Arbacia* sperm. The present study tests this possibility using antibodies dissociated from *Echinarachnius* sperm.

Arbacia sperm and *Echinarachnius* sperm were treated with whole anti-*Arbacia* sperm serum and control serum. The treated sperm were washed until the washings did not agglutinate *Arbacia* sperm, after which the sperm were fixed in 1% formalin. Subsequently, the antibodies bound to the sperm were dissociated off by treatment with pH 2.5 sea water at 37° C., for 12 hours and separated from the sperm by centrifugation. Following dialysis against sea water, the dissociated antibodies agglutinated homologous sperm. The dissociated antibodies were converted to the nonagglutinating, univalent form by papain digestion (Porter, 1958).

Four experiments were performed involving two separate dissociated antibody samples. Antibody from *Arbacia* and *Echinarachnius* sperm were adjusted to equivalent *Echinarachnius* sperm-agglutinating titers prior to papain digestion. The antibodies removed from *Echinarachnius* sperm failed to reduce the fertilizing capacity of *Arbacia* sperm, whereas antibodies removed from *Arbacia* sperm significantly reduced the fertilizing capacity of *Arbacia* sperm. From this result, it is concluded that the antigens shared with *Echinarachnius* sperm have no significant role in *Arbacia* fertilization.

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August 24, 1963

Changes in the polysome composition following fertilization in eggs of the sea urchin, Arbacia punctulata. Wayne Daugherty.

Previous studies demonstrated that aggregated ribosomes (polysomes) are involved in protein synthesis. Polysomes were prepared by the method of Monroy and Tyler (1963). A post-mitochondrial supernatant was placed on a 24-ml. 15–30% linear sucrose gradient. After centrifugation (55,000 *g*, two hours), 24 one-ml. fractions were collected (no. 1 being the heaviest). Optical density (260 mμ) plotted against the distance along the sucrose gradient showed six peaks, by position, the slowest sedimenting fraction (no. 18, peak A) corresponding to the unaggregated ribosome peak, the five heavier peaks (B, C, D, E, F: fractions 15, 12, 9, 6, 3, respectively) to the aggregated ribosomes as described by Monroy and Tyler. Preliminary electron microscope observations of both A and pooled C, D, and E indicate that A is the monosome peak, whereas the heavier peaks represent the polysomes. During development, changes were observed in the relative areas under the six optical density peaks. In the unfertilized egg, the size of the peaks decreased in the order A, B, C, F, D; peak E was absent. At 13 hours of development, the size of the peaks decreased: A, B, D, C, F, E. The pattern continued to change, the decreasing order observed at the gastrula stage (17 hours) being D, A, B, E, F, C. Pulse labeling (8 minutes, ^{14}C-valine) of 18-hour embryos resulted in higher radioactivity in the polysomes (fractions 3, 6, 9, 12, and 15) relative to the monosomes (fraction 18). After a 10-minute chase, the radioactivity completely decayed in fractions 3, 6, 9, and 12, but remained almost unchanged in 15 and 18, and became higher in the soluble protein region. Changes observed in ribosome aggregation following fertilization are presumably related to the increasing rate of protein synthesis. This aggregation is perhaps governed by the interaction of free ribosomes and messenger RNA.

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Myokinase activity in sea urchin egg fragments. Barbara A. Horwitz and Leonard Nelson.

The equilibrium constant of the reversible reaction catalyzed by myokinase ($2\ ADP \rightleftharpoons ATP + AMP$) is close to unity, suggesting that this enzyme may be important in maintaining a relatively constant level of ATP in metabolizing cells. Homogenized eggs of *Arbacia punctulata*, as well as homogenized nuclear thirds and nuclei, were examined for the presence of myokinase.

Nuclear thirds were obtained by centrifugation of intact eggs at 30,000 *g* for 45 minutes, thus eliminating the pigment and heavy yolk granules and most of the mitochondria. Centrifugation of the homogenized thirds on a sucrose gradient yielded nuclei, most of which possessed granular material adhering to the nuclear membrane. Electron microscopic examination of this nuclear fraction revealed few recognizable mitochondria. These preparations were incubated for one hour at room temperature in the Bendall medium (1954) and inorganic phosphate was measured.

Myokinase activity was observed in all three preparations analyzed, the total activity (μg. P_i/ml. preparation) being lowest in the homogenized eggs and highest in the nuclear thirds. When aliquots of homogenized eggs were boiled and then centrifuged to remove the denatured protein, the myokinase activity was twice as high as that of similarly centrifuged, unheated aliquots and was comparable to that of the nuclear thirds. Heating the nuclear thirds did not increase the total activity. These results suggest that the eggs contain an enzyme which may interfere with myokinase and which may be removed upon heating or fractionation. The similar total activities seen in the thirds and the heat-treated fractions of homogenized eggs implied that the mitochondria did not contribute significantly to the myokinase activity. The specific activities of the three preparations (μg. P_i/mg. protein) followed the same relationship as the total activities with respect to order of magnitude. The ratios of total to specific activity in the nuclear fraction and the nuclear thirds did not differ significantly, indicating a loss of highly active material from the nuclear fraction. Further studies are planned to determine the distribution of this enzyme and its importance in development.

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Splitting of sialo-compounds by sea urchin sperm. ROY PETRIE AND LEONARD NELSON.

The sialic acids have been found in large quantities in the gametes of a wide variety of organisms and may play a role in fertilization. In order to clarify this role, an investigation of enzymes which act on sialo-compounds was undertaken (such enzymes have been demonstrated in bacteria, viruses, rat liver and brain, and human plasma).

Assays for a neuraminidase in *Arbacia punctulata* sperm and eggs were made. Whole eggs, whole sperm, homogenated sperm, homogenated eggs, seminal fluid, and egg suspension fluid were incubated with N-acetylneuramin-lactose, a neuroaminidase substrate, at 23°, 37°, and 40° C., at pH 5.1, 6.0, 7.2 and 7.6 for 15-, 30-, 45-, and 60-minute periods. Unbound sialic acid was determined by Warren's thiobarbituric acid method. Homogenated sperm decreased the amount of unbound sialic acid normally found in such homogenates, rather than cause the increase that would be expected from neuraminidase activity. No activity was observed in the other preparations.

The unexpected decrease in unbound sialic acid caused by the sperm homogenate could have been due either to a binding or an enzymatic breakdown of unbound sialic acid. However, since acid hydrolysis of the sperm homogenate assay material did not further release any unbound sialic acid, the possibility of an enzymatic breakdown of unbound sialic acid was considered.

Incubation of free sialic acid with a sperm homogenate supernatant at 37° C. and pH 7.2 reduces the amount of substrate by 25 to 50%. Heating the crude enzyme preparation in boiling water for 10 minutes reduced the activity approximately 10 to 25%. Attempts to isolate the enzyme by precipitation with ammonium sulfate have been unsuccessful.

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Alkaline phosphatase activity in the embryo of Arbacia punctulata. RONALD J. PFOHL AND ALBERTO MONROY.

The activity of the alkaline phosphatase enzyme (μg. P_i/mg. protein/hour from DL-β-glycerophosphate) in whole homogenates of various developmental stages of *Arbacia punctulata* was as follows: (1) unfertilized eggs, 1.5; (2) late gastrula and prism, 4.7; (3) early pluteus, 7.0; (4) mid pluteus, 9.4; (5) advanced pluteus, 10.2. The pH optimum was above 9.0.

Treatment of homogenates of unfertilized eggs with butanol (Morton, 1954) resulted in a doubling of the activity. In the advanced pluteus stage, on the other hand, treatment of homogenates with butanol produced no change. The supernatant obtained upon centrifugation of the untreated homogenate, however, contained little or no activity. Apparently, in the pluteus, the enzyme, though bound to lipoprotein particles, is in an active state and is released into solution upon treatment with n-butanol.

Fractionation with $(NH_4)_2SO_4$ at 50% and 100% saturation had shown the enzyme to be confined to the 100% fraction (Pfohl and Monroy, 1962). The activity of the 100% fraction from the dialyzed butanol extracts was as follows: (1) 15.7; (2) 17.3; (3) 22.3; (5) 89.3.

The increase in the enzyme activity in the whole homogenates suggests a release of inhibition during the course of development. Butanol treatment, dialysis and $(NH_4)_2SO_4$ precipitation may remove the inhibitor(s) to yield the full complement of enzyme activity. The marked rise in activity in the advanced pluteus may be due to *de novo* synthesis of the enzyme. The total activity at this stage was about six to seven times greater than the activity in the unfertilized egg. Investigations are under way to determine the relative contributions of *de novo* synthesis, release of inhibition and/or an activation of precursor molecules to the increase in enzyme activity during development.

Aided by Training Grant 5T1GM998-02 from the National Institutes of Health.

Effects of actinomycin-D on the rate of protein synthesis during the development of Arbacia punctulata. KIRBY D. SMITH.

Eggs of *Arbacia punctulata* were cultured in sea water containing 15 μg. of actinomycin-D per milliliter, beginning one-half hour prior to fertilization. At various times during development, aliquots of embryos were taken from the solution and incubated for 10 minutes in a solution containing radioactive amino acid (S^{35}-methionine or C^{14}-valine). The incorporation was stopped by adding a large excess of non-radioactive amino acid. The total uptake and the incorporation of amino acid into protein were determined by the method of Mans and Novelli.

In control embryos there are two periods of rapid uptake and incorporation of amino acid into protein. The first period extends from the time of fertilization to the 16-cell stage. Following this stage there is a decline in both the rate of uptake and the rate of incorporation into protein until the beginning of gastrulation when the second period of rapid uptake and incorporation begins.

Embryos treated with actinomycin-D show an increase over the controls in both the rate of uptake and the rate of incorporation during the first period, while the increased rate at gastrulation is completely inhibited. These results would tend to indicate that the first period of active protein synthesis is not dependent upon newly synthesized messenger RNA, while that at gastrulation is. However, if a comparison of the ratio of amino acid incorporated into protein to the total amount of amino acid taken up is made between the control embryos and those cultured in actinomycin, a definite inhibition of protein synthesis is seen in the embryos treated with actinomycin. A complete understanding of these results awaits an analysis of the amino acid pool. This analysis is being carried out at the present time.

It was also shown that actinomycin must be present at the time of gastrulation in order to inhibit development.

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Glycogen and acid-soluble phosphorus in the female reproductive tract of the smooth dogfish. WILLIAM THORNTON.

Mustelus canis is a viviparous elasmobranch having a gestation period of 10 to 11 months, the last two-thirds of which are characterized by a yolk-sac placenta. Since ovulation and fertilization occur in June, the mature animal, as found in Woods Hole during the summer, is in a pre-placental state.

Thus far there is no evidence to suggest that the developing embryos receive any nutrients from the mother during the pre-placental period; it is assumed that the egg yolk serves this function. Data exist on the water, organic matter and ash content of the ova and early embryos of this species (TeWinkel, 1963).

In the present study, an attempt was made to describe the glycogen, inorganic phosphorus, and acid-soluble phosphorus content of the reproductive tract, egg case, yolk, and embryo (which ranged from 2 to 4 cm. in length). The results represent averages of 10 animals, glycogen being expressed in g./100 g. tissue, wet weight, phosphorus as mg./100 g. tissue, wet weight. The endometrium, oviducal glands, and oviducts contained 0.09, 0.13, and 0.07 g./100 g. of glycogen, respectively. The yolk and egg cases contained 0.03 and 0.02 g./100 g. of glycogen, and the embryos 0.04 g./100 g. Phosphorus determinations showed that the endometrium, oviducal glands, and oviducts contained 11, 16, and 15 mg./100 g. of inorganic, and 17, 29, and 11 mg./100 g. of acid-soluble organically-bound phosphorus. The yolk and egg case contained 5 and 1 mg./100 g. of inorganic, and 33 and 3 mg./100 g. of acid-soluble organic phosphorus, respectively. Scarcity of experimental material did not allow phosphorus determinations in the embryo.

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Uptake of C^{14}-glucose by unfertilized and fertilized eggs of Asterias forbesii. HELEN TOLIS AND ALBERTO MONROY.

The rate of uptake of C^{14}-D-glucose and incorporation of C^{14} into proteins was studied in relation to maturation and fertilization in *Asterias forbesii* eggs. Eggs were incubated for 30 minutes in C^{14}-D-glucose (1 μC./ml.) and uptake was stopped by addition of non-radioactive D-glucose. Total radioactivity and incorporation were estimated (Mans and Novelli, 1960). Total nitrogen was used as a reference. *Asterias* eggs begin to mature when shed in sea water and are fertilizable in the course of maturation. The rate of uptake in unfertilized eggs declined rapidly during maturation and continued to decrease after maturation. In fertilized eggs an initial decrease (smaller than in the unfertilized eggs) in rate of uptake of C^{14}-glucose was followed by an increased uptake after formation of the second polar body. Experiments with the Mediterranean *Asterias* have shown that eggs utilize C^{14} from glucose for synthesis of alanine, glutamic, and aspartic acids. In the present study, a small incorporation of C^{14} into proteins was observed during maturation both in unfertilized and fertilized eggs. After completion of maturation, the rate of incorporation remained constant in the unfertilized eggs but rapidly increased in the fertilized eggs. Unfertilized eggs, following spontaneous or deliberate (hypertonic sea water) artificial activation, showed a considerable increase of the rate of uptake over that of the unfertilized eggs. This was not accompanied by any significant increase in incorporation into proteins.

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Uptake and incorporation of radioactive amino acids in unfertilized and fertilized eggs of Asterias forbesii and Spisula solidissima. HELEN TOLIS AND ALBERTO MONROY.

The rate of uptake of S^{35}-L-methionine and of L-valine-1-C^{14} and their incorporation into proteins was studied in unfertilized and fertilized eggs of *Asterias* and *Spisula.* Eggs were incubated for 5 minutes in the labelled amino acid (1 μC./ml.) and uptake was stopped by dilution with cold amino acid. Total radioactivity and incorporation were estimated (Mans and Novelli, 1960). Total nitrogen was used as a reference. Maturation of *Asterias* eggs is initiated upon shedding in sea water, independently of fertilization. In *Spisula* eggs, on the contrary, maturation is initiated by fertilization. In *Asterias* eggs, there was practically no difference in the rate of uptake of the two amino acids between unfertilized and fertilized eggs until after the formation of the second polar body. Following completion of maturation, the rate of uptake underwent a rapid increase in the fertilized eggs; at the 2-cell stage it was at least twice as great as in the unfertilized eggs, and continued to increase. In *Spisula,* no change occurred in rate of uptake as long as the eggs remained unfertilized. But after fertilization-induced breakdown of the germinal vesicle a very rapid increase began. At the 2-cell stage the rate of uptake was at least 50 times greater than that of the unfertilized eggs. A slight incorporation into proteins occurred during maturation, both in the unfertilized and fertilized eggs of *Asterias.* Following completion of maturation, however, the incorporation dropped in the unfertilized eggs and rapidly increased in the fertilized ones. In *Spisula,* the rate of incorporation followed much the same pattern as the uptake.

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Occurrence of lactic dehydrogenase in starfish sperm. JAMES WALLINE AND LEONARD NELSON.

Mammalian sperm exhibit lactic dehydrogenase (LDH) isozyme patterns when examined electrophoretically (Goldberg, 1963). Starfish sperm, which carry on respiratory metabolism, in contrast to the primarily glycolytic metabolism of mammalian sperm, were subjected to disc electrophoresis. Washed sperm were suspended in 10 ml. of 0.05 *M* Tris buffer, pH 8.3, and sonicated for three minutes in an ice bath. After centrifugation at 80,000 *g* for 90 minutes, the supernatant was precipitated with 60% ammonium sulfate, resuspended in the buffer and dialyzed for 12 hours. One-tenth ml. of the partially purified extract, containing approximately 1 mg. of protein, was added to the columns and a current of 2.5 milliamperes per column was applied at room temperature.

Unfixed gels were rinsed in cold 0.05 *M* Tris buffer, pH 8.3, and incubated at 37° C. for 4–8 hours in a reaction mixture containing sodium lactate, NAD, nitro blue tetrazolium, 0.05 *M* Tris buffer, pH 8.3, and phenazine methosulfate. Sites of LDH activity in the gels were localized by the precipitation of the reduced tetrazolium salt. Comparison was made to control gels having no lactate in the reaction mixture. The experimental and control gels had three bands in common, while the lactate-containing gels showed 1–2 additional bands, indicating that sperm extracts contained a small amount of endogenous reducing substance as well as an apparently specific LDH.

Utilization of lactate by living sperm could not be detected by the Barker-Summerson method; however, sperm extracts oxidized NADH in the presence of excess pyruvate. The decrease in absorption at 340 mμ measured spectrophotometrically appears to follow the kinetics of a first order reaction.

While a substance which acts on lactate appears to be present in starfish sperm, the establishment of a definite isozyme pattern may depend on further purification of the sperm extract.

This study was aided by Training Grant 5T1GM998-02 from the National Institutes of Health.

GENERAL SCIENTIFIC MEETINGS

AUGUST 26–29, 1963

Abstracts in this section are arranged *alphabetically by authors.* Author and subject references will also be found in the regular volume index, appearing in the December issue.

PAPERS READ

Locomotion and cytoplasmic streaming in Allogromia sp. strain N.F. R. D. Allen, W. R. Pitts, S. Lloyd and T. Reynolds.

Establishment and withdrawal of the reticulopodial network of the naked marine foraminiferan, *Allogromia* sp. strain N.F. (from Dr. John Lee of N.Y.U.) have been studied by direct observation and by detailed frame-by-frame analysis of cinemicrographic records taken at 8 and 16 frames per second. The primary processes involved in network activities appear to be pseudopodial extension, attachment to the substratum, branching, splitting, fusion, and retraction.

Cytoplasmic streaming is typically bidirectional in all parts of the network except in excised portions, in which unidirectional streaming may also occur. Some particles move at uniform velocities over distances of the order of 100 microns; others change velocity or direction abruptly, often at attachment points.

An important and neglected aspect of pseudopod formation and retraction is the conversion of fibrillar pseudopodial substance into "droplets" which may be transported to another part of the network and converted anew into fibrillar material. The conversion of fibrillar to droplet material can occur in less than a second on mechanical injury or on contact with forams of different species; it can also occur more slowly, *e.g.,* in gradually degenerating excised portions.

While it is too early in the fact-gathering stage to propose a definitive model for the mechanism of streaming in forams, an alternative type of mechanism can be suggested to replace the active-shearing hypothesis of Jahn and Rinaldi (1959), which has received negative support in a recent electron microscopic study of Wohlfarth-Botterman (1961). The proposed type of mechanism requires multiple sites for application of the motive force. Motion analysis data suggest local contractions may occur at pseudopodial tips and attachment points.

Supported by U.S.P.H.S. Grant GMO 8691.

Anomalous rectification in the squid giant axon injected with tetraethyl ammonium chloride. Clay M. Armstrong and Leonard Binstock.

The voltage clamp technique was applied to the study of the ionic currents in a squid giant axon injected with TEA. As observed by Tasaki and Hagiwara, the action potential is prolonged, and there is little or no outward current under voltage clamp, even with cathodal voltage steps (*i.e.,* inside is made more positive) two seconds long. Membrane conductance in the steady-state during a cathodal voltage step is approximately twice that of the resting membrane. Voltage steps applied to an injected axon resting in 100 or 440 m*M*

K produce large currents which rectify anomalously, *i.e.*, inward current flows more readily than outward, by a ratio of as much as 40:1. (An uninjected nerve under the same conditions rectifies slightly in the same direction.) These currents are essentially the same in 100 m*M* and 440 m*M* KCl, and are unchanged by substituting Na-isethionate for NaCl in the 100 m*M* K, indicating that the currents are largely K. Application of a large anodal voltage step to an axon resting in 440 m*M* K results in an approximately exponential decrease of membrane conductance. Following termination of the anodal pulse, the membrane conductance increases with an S-shaped time course, qualitatively like the K conductance of Hodgkin and Huxley. The Na currents in the injected axon behave qualitatively as they do in the uninjected axon.

The findings may be summarized by saying that the potassium channels of the injected axon have a one-way valve discriminating against outward current in series with the voltage valve of the normal axon.

Localization of phosphatases in Arbacia eggs during first cleavage. WALTER AUCLAIR,[1] JERROME MANGAN AND DOUGLAS MARSLAND.[2]

The localization of four phosphatases, acid and alkaline GPase (beta-glycerophosphatase) and acid and alkaline ATPase (adenosine-triphosphatase), was determined in whole eggs by modifications of the lead and cobalt techniques of Gomori. Fixatives used were either formol-saline (2° C., two hours) or ethanol (50%, −12° C., one hour). A moderate pressure-centrifugation (6000 p.s.i., 41,000 *g*, 1–2 minutes, 25 minutes post-insemination) preceded fixation, and untreated, cleaving eggs were used as controls. Fixation of centrifuged eggs was: (a) immediate; (b) 5–10 minutes later; or (c) at the incidence of furrowing.

The formalin- and ethanol-fixed eggs showed a similar localization of enzyme activity, except that the formalin eggs were negative for acid ATPase, indicating inhibition. An almost identical distribution was found for the two alkaline phosphatases, suggesting non-specificity of alkaline ATPase. The greatest concentration of the alkaline phosphatases and of the acid ATPase in the dividing control eggs occurred in the mitotic figure, whereas acid GPase activity appeared as a dense granular zone bordering the spindle-aster region.

Eggs, fixed either immediately or shortly after pressure-centrifugation, showed a high concentration of the alkaline phosphatase and of acid ATPase in the hyaline zone; acid GPase activity was concentrated in a dense band at the boundary between the yolk and hyaline zones. Later, at cleavage, acid ATPase appeared within the mitotic apparatus, which was displaced into the hyaline zone. Also, a redistribution of the acid GPase activity was observed as stained granules within the asters.

Specificity of the ATPases was tested by incubation with ADP, muscle adenylic acid, ATP and 10^{-3} *M* PCMB (p-hydroxymercuribenzoate), and absence of substrate. Only acid ATPase was specific, and was inhibited by PCMB.

Differences in the persistent metabolic rhythms of fiddler crabs from two levels of the same beach. FRANKLIN H. BARNWELL AND FRANK A. BROWN, JR.

During June, July, and August fiddler crabs of two species, *Uca pugilator* and *Uca pugnax*, were collected from the upper and lower regions of the intertidal zone at the Chapoquoit marsh near Woods Hole, Mass. Within two or three hours of collection male crabs of approximately the same size were placed in continuous recording respirometers. While the crabs were maintained in constant low illumination at 23.9° C. their rates of oxygen consumption were followed for periods ranging from two to five days. For each species, crabs from the lower region of the intertidal zone had a higher mean rate of metabolism than crabs from the upper region. When crabs from the same level of the beach were compared, the mean metabolic rate of *U. pugilator* was higher than the mean rate of *U. pugnax*.

In each of six experiments a persistent tidal rhythm of metabolic activity was present. In crabs of both species from the upper level of the beach, maximum tidal activity occurred from one-half to 4 hours before it occurred in crabs from the lower level. Crabs from the upper level of the beach often showed a pronounced increase in amplitude of the tidal peak occurring at night, suggesting that a large, persistent, 24-hour component consisting of nocturnal activity, was present in addition to the tidal component. In some cases it was observed that the daytime tidal peak did not fall exactly between the nocturnal tidal peaks but was displaced to an earlier time of day. Several trips to the salt marsh in July near the time of new moon when low tide occurred between 10 PM and 2 AM confirmed that both *U. pugilator* and *U. pugnax* exhibit nocturnal activity.

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The effect of temperature on the in vitro coagulation of the blood of the smooth dogfish Mustelus canis and the sea bass Centropristes striatus. FRANK A. BELAMARICH, ROSEMARY W. ESKRIDGE AND DOUGLAS M. SURGENOR.

Both the sea bass and smooth dogfish exhibit optimal clotting at calcium levels high above their physiological concentrations. Citrated whole blood from sea bass and dogfish at final calcium levels of 25 m*M* and 67 m*M*, respectively, clotted quite quickly at temperatures from 0–45° C. At near physiological calcium concentrations (8 m*M*), clotting times were lengthened considerably. Under these conditions dogfish blood clotted more slowly and sea bass more rapidly than human blood.

The temperature optimum of sea bass citrated whole blood was unaffected by high calcium concentration, while citrated and uncitrated dogfish whole blood exhibited different optima at high and low calcium levels. The optimum for low calcium concentration was near 35° C. in contrast to 15° C. for high calcium. An analysis of plasma and cellular components revealed red cells to be a major contributor to this phenomenon.

With the addition of aqueous skin extracts, clotting times in both animals were shortened considerably at both high and low calcium levels. In a system adjusted to give similar clotting times at 40° C., the dogfish exhibited more rapid clotting than the sea bass from 5–40° C. At 0° C., however, sea bass blood clotted more rapidly. In the presence of skin extract, temperature optima for the dogfish remained unaffected by high calcium concentration.

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The binding of aldehyde in the in vitro bacterial luciferase reaction. DERIC BOWNDS, LINDA GARRICK, RICHARD MUESING, JAMES SPUDICH AND J. W. HASTINGS.

Bacterial luciferase from *Achromobacter fischeri* requires $FMNH_2$, oxygen, and a long chain aldehyde for light emission, the reaction sequence being in that order. The nature of aldehyde binding in the final reaction step was studied in these experiments. To determine whether or not aldehyde binding is reversible, the reaction was carried out in the presence of two different aldehydes (decanal and dodecanal), which by themselves result in characteristically different velocity constants in the reaction. If the binding of aldehyde were irreversible, then a biphasic reaction characteristic of the two different aldehyde-enzyme components should result. The kinetics demonstrate, however, that the binding is fully reversible; a monophasic reaction with an intermediate velocity constant was observed.

This result predicted that a compound which binds with enzyme at the aldehyde site should, when added during the course of the reaction, react readily with free enzyme. A variety of compounds which were presumed to be capable of mimicking aldehyde in binding (yet unable to react chemically in the luminescent reaction) were therefore tested by adding them to a reaction in progress. Several effective compounds were discovered: 2-decenal, 2,4, hexadienal, benzaldehyde, cinnamaldehyde, undecanone, decyl acetate, gamma-decalactone, decanol, and 1-decenol. A number of compounds gave little or no indication of binding: decane decanoic acid, 1-decenoic acid, decyl thiocyanate, thiodecane, 1-decene, decyl chloride and decyl bromide. Although we have studied only a limited number of compounds, it appears that compounds which have more than four carbon atoms and a terminal oxygen atom can occupy the binding site of aldehyde on the luciferase enzyme.

Enzyme patterns in eggs, developing embryos and adult tissues of Spisula solidissima. HELEN B. BURCH AND PHILIP STRITTMATTER.

To extend earlier studies on terminal electron transport in developing embryos of *Spisula solidissima,* the patterns of flavoproteins, dehydrogenases and hydrolytic enzymes in eggs, developing embryos and adult tissues have been examined. The levels of the flavoproteins in unfertilized eggs were compared to those of adult liver, because the flavin content of liver (0.015 μmoles per gm. tissue) is two-fold higher than in unfertilized eggs or adult heart and gill. Furthermore, almost 90% of the flavin in liver is FAD, compared to 80% in the other tissues. The higher content of flavin in liver is reflected in a two- to four-fold increase in D-amino acid oxidase, xanthine oxidase and glycolate oxidase activities. Glutathione reductase, in contrast, is two-fold higher in egg homogenates. Of the large group of flavin enzymes tested, aldehyde oxidase appears to be the only enzyme activity which is high in liver but undetectable in unfertilized egg homogenates. This enzyme, which may represent one example of *de novo* enzyme synthesis during differentiation in this species, is still not detectable in two-day-old embryos that have a functioning liver and intestinal tract. No significant differences in malic dehydrogenase and TPN isocitric dehydrogenase activities in egg and liver homogenates were observed, and the ATP, ADP and AMP nucleotidase activities are approximately 0–400% higher in liver homogenates. Heart succinic dehydrogenase, as shown previously, and the liver aldehyde oxidase thus represent the only two enzymes of a large number of oxidative and hydrolytic enzymes that have been found in much higher levels in adult tissues than in eggs or early embryos.

Some morphological and physiological effects of thalidomide on the embryos of Fundulus heteroclitus. JOSEPH A. BURKE AND JOSEPH F. SPEAR.

Thalidomide has been shown to affect human embryos. In the present study the vertebrate embryo of *Fundulus heteroclitus* was used to ascertain effects on sperm viability, heart rate, and growth rate.

Stock solutions of thalidomide (2 mg./ml.) were made by dissolving 50 mg. in 5 ml. of 0.1 N NaOH, neutralizing with 1.5 ± 0.1 ml. of 0.1 N HCl, and adding sea water to 25 ml. The pH of all solutions was adjusted to 6.0.

Sperm were shed into 2 mg./ml. and 1 mg./ml. thalidomide and left for one to four minutes. Eggs were stripped into these suspensions. Within one-half hour the eggs were transferred and grown in sea water at 20° ± 1° C. with daily changes. Sperm exposed to 2 mg./ml. thalidomide averaged 21.5% successful fertilizations, 1 mg./ml. thalidomide 59.0%, and controls 77.0% successful fertilizations.

Eggs, normally fertilized, were placed at the 32-cell stage in 1 mg./ml., 0.5 mg./ml., and 0.1 mg./ml. thalidomide. Solutions were changed every 24 hours. No growth inhibition was noted in undechorionated embryos. The chorions of eggs at early gastrula were pierced in both experimentals and controls. Under similar experimental conditions, definite growth inhibition occurred, especially in the pectoral fin.

Dechorionated and undechorionated embryos with pulsating hearts were grown in daily changes of 1 mg./ml., 0.5 mg./ml., and 0.1 mg./ml. thalidomide. No effect on heart rate was noted. In embryos grown from middle gastrula in 1 mg./ml., 0.5 mg./ml., and 0.1 mg./ml. thalidomide, heart rates were faster than controls in proportion to solution strength. After 24 hours in 1 mg./ml. and 0.5 mg./ml., embryo hearts beat slower than controls up to a point 10 days later when both coincided with controls. Embryonic hearts in 0.1 mg./ml. beat faster than controls for the duration of the experiment.

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Metachronal ciliary coordination in ATP-reactivated models of Modiolus gills. FRANK M. CHILD AND SIDNEY TAMM.

So-called "models" of the ciliated lateral cells of the gill filaments of the ribbed mussel, *Modiolus demissus* Dillwyn, can be prepared by (1) extracting the amputated filaments in a solution (ES) consisting of 40% (v/v) ethylene glycol, 10% (v/v) glycerol, 0.1 *M* KCl, 0.01 *M* phosphate buffer, pH 7.5, at 0° C., for at least 1.5 hours; (2) washing the extracted filaments in a solution (WS) consisting of 0.1 *M* KCl, 0.005 *M* $MgCl_2$, 0.01 *M* histidine, 0.01 *M* phosphate buffer, pH 7.5 at 0° C. for two minutes; and (3) reactivating the washed filaments in a solution (RS) consisting of 0.003 *M* adenosine triphosphate (ATP) in WS, initially at 0° C., but warmed to 23–26° C. as the preparation is observed by phase-contrast microscopy.

The lateral cilia do not beat in ES, nor do they beat in WS. In RS, however, they beat rhythmically, with a form of beat similar to living cilia. The patterns of ciliary coordination vary between specimens, between filaments and between regions of the same filament. Preparations showing good coordination appear to be those in which tissue maceration has been minimized. In these we observe the following range of coordinated activity: (1) normal laeoplectic metachronal waves, with wave-lengths and wave-velocities approaching those in living gills, *i.e.,* pluricellular metachrony, (2) unicellular metachrony, (3) unicellular synchrony, and (4) occasional dexioplectic pluricellular metachrony. Cinematographic records of these phenomena have been obtained.

In view of the well known impairments of metabolic and membrane-associated properties of "cell models," hypotheses on the mechanism of metachronal wave conduction will need to involve structures and processes demonstrable in the "models" as well as in the living cells.

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Fluorescence properties of Loligo pealei hemocyanin. JORGE E. CHURCHICH.

The present abstract will be confined to the effect of pH upon the fluorescence and phosphorescence properties of the proteins hemocyanin and apohemocyanin.

It has been found that the fluorescence efficiency of hemocyanin is invariable between pH 5 and pH 6.8. At alkaline pH the fluorescence efficiency is considerably decreased and the quenching is in accord with the increase in the absorption band at 350 mμ.

Removal of the copper by dialysis against 10^{-2} *M* KCN enhances three times the fluorescence yield. The spectral overlap of the absorption band of the oxygenated copper with the emission band of tryptophan leads to the prediction that non-radiative energy transfer should increase in importance with increasing extent of oxygenation.

Phosphorescence experiments were undertaken at 77K in liquid nitrogen and the protein solutions prepared in propylen-glycol 50%–water 50%. The mean lifetime in the excited state has been measured and the values $\tau = 3.6$ and $\tau = 5$ seconds were obtained for hemocyanin and apohemocyanin, respectively. Apparently conversion from the triplet state is favored by the presence of copper.

The quenching of the triplet state requires exchange interaction; therefore a close contact between copper and tryptophan in the hemocyanin is suggested.

Polarization of fluorescence of hemocyanin conjugated with DNS was made between the range of 4° C. and 35° C. From a plot of polarization of fluorescence versus T/η (temperature over viscosity), the relaxation time at 25° C. was evaluated ($\rho h = 0.7 \times 10^{-7}$ sec). The value is too small for a protein of molecular weight of the order of 4,000,000; and the result is interpreted to mean the presence of loosely connected subunits possessing some rotational freedom with respect to one another.

A pulsatile pressure transport system across artificial membranes. C. LLOYD CLAFF AND ARMAND A. CRESCENZI.

Heretofore most attempts to oxygenate blood by diffusion of gas through a membrane have been done with laminar flow and steady pressure. Many months of experimenting convinced us that no amount of redesigning would overcome the inherent problems of this system, *i.e.,* two parts of the stream flow the entire length of the interphases, subjected to all the activities of interphase, and the middle of the stream may receive less than adequate oxygenation. We have used pulsatile pressure with intermittent flow to create new interphases at each pulse, creating alternate positive and negative pressures, bringing about a condition where the difference in partial pressures of the gas phases and blood is greatest when the blood stream is the thinnest. Pulsatile pressure with its intermittent flow, combined with "encapsulated elasticity," *i.e.,* confining the stretch of the membrane bag within a predetermined limit of ten-thousandths of an inch by restraining plates, accurately positioned, has given us a means of control of volume, regulation of gradient pressures, control of surface areas, and finally, by incorporating an internal heat exchanger, homeothermic temperature control, without extra blood priming volume. Five and a half square meters of Teflon membrane oxygenate the same volume of blood to saturation (4000 cubic centimeters per minute) as do 480 square meters of human lung used in resting metabolism, *i.e.,* 20% of the lung area. The assembly becomes a "building block" for artificial organs, such as a heart-lung combination, or artificial kidney. It also provides a large area for irradiation. Priming volume is 10% of the flow rate, so that the system is particularly applicable to extracorporeal oxygenation of blood for newborn and premature babies, and limited perfusion in cancer chemo-therapy.

Effects of micromere deletion on development in Ilyanassa. ANTHONY C. CLEMENT.

Each of the following micromeres has been removed microsurgically from the *Ilyanassa* egg and the developmental effect observed: 1a, 2a, 2b, 2c, 2d, 3a, 3b, 3c, 3d, and 4d.

The loss of 1a results in absence of the left eye and probably a slight reduction in size of the left velum. The effect of removal of each of the second quartet micromeres is different. Loss of 2a affects the development of the left velum and, usually, also the development of the left eye and left statocyst. Loss of 2b produces little or no effect on development. Loss of 2c results in severe defects; these include eversion of the stomodeum, reduction in size of the shell, and absence of the heart. Loss of 2d result in absence of the shell, or its severe reduction. The effect of removal of each of the third quartet micromeres is also different. The loss of 3a results usually in a slight reduction in size of the left velum. The loss of 3b tends to reduce slightly the size of the right velum. The loss of 3c results in absence of the right statocyst and right half of the foot. The loss of 3d results in absence of the left statocyst and left half of the foot. As previously reported, removal of 4d results in absence of the intestine and heart. Each of the micromeres studied appears to have a different developmental value.

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Studies of the structure of Loligo pealei hemocyanin. I. Subunit molecular weights and dissociation equilibria near neutral pH. L. B. COHEN AND K. E. VAN HOLDE.

In the pH range 4.6 to 10.6 the hemocyanin from *Loligo pealei* has three easily identifiable components with sedimentation coefficients of 56, 19, and 10S, the relative amounts of each depending on pH, temperature, and magnesium ion concentration. Light scattering gives molecular-weight values of 3,700,000 for the 56S, 675,000 for the 19S, and 385,000 for the 10S. Sedimentation equilibrium gives an M_z of 3,400,000 for the 56S, an M_w of 680,000 for the 19S, and an M_w of 395,000 for the 10S. The 56S and 10S species appear to be homogeneous, but the 19S species shows a small amount of trailing material in a sedimentation velocity experiment.

From the above results, the molecular-weight ratio of 56S to 19S is about 5, and the 56S to 10S ratio is about 10. We have therefore concluded that the 56S material first breaks into fifths and that the fifths then break in half.

The reassociation of fifths into wholes has been followed over the pH range 5.5 to 7.0 in the absence of divalent cations. The optimum pH for reassociation is found to be near 5.8. At pH 7.0 no reassociation has taken place after two days at room temperature. At pH 5.5, before any reassociation has occurred, the fifths are disrupted into species with different sedimentation coefficients. It is not clear whether this maximum at pH 5.8 is a maximum in rate or is due to changes in the equilibrium position with changes in pH. The time course of the reassociation from fifths has been studied, and it appears that the kinetics may be of order higher than three.

The wholes-fifths equilibrium is temperature-dependent; at pH 5.8 at 2° C., a solution contains mainly fifths, while at 25° C. the equilibrium lies on the side of wholes.

Studies of the structure of Loligo pealei hemocyanin. II. Dissociation equilibria in alkaline solution. K. E. VAN HOLDE AND STEPHEN C. HARRISON.

In alkaline solution, the hemocyanin of *Loligo pealei* can exist either as whole molecules ($s_{20,w} = 56S$), as fifths ($s_{20,w} = 19S$), or as tenths ($s_{20,w} = 10S$). The particular species found is a function both of pH and of magnesium ion concentration. The present investigation is concerned with two problems: (1) the Mg^{++} and temperature dependence of the 56S–19S equilibrium, and (2) the pH dependence of the 19S–10S equilibrium.

(1) Blood diluted directly into pH 8.2 buffer yields primarily 19S subunits. Addition of Mg^{++} to a concentration of 0.01 *M* at 27° C. produces association into 56S molecules, as observed both by light scattering and by sedimentation velocity. The dependence of the equilibrium point on Mg^{++} concentration at pH 8.2, 30° C. has been examined by light scattering, and the results indicate the binding of about 10 Mg^{++} per 56S molecule in the 19S to 56S association. The 56S–19S equilibrium also exhibits a marked temperature dependence; in the range 30° C. to 40° C., dissociation is favored by increasing temperature.

(2) The 19S–10S equilibrium has been studied both by light scattering and by sedimentation velocity. The fraction of 19S material present at various pH is consistent with the following model: (i) 19S dimer is in equilibrium with a deprotonated form in which *two* hydrogen ions have been split off; (ii) the deprotonated dimer is in equilibrium with two monomeric (10S) subunits. Schemes involving one or three hydrogen ions do not fit the data. The mean pK_a for the two dissociations in the dimer–deprotonated dimer equilibrium is about 8.9. The data also indicate that boundaries in the ultracentrifuge with sedimentation coefficients intermediate between 10S and 19S represent rapid equilibria of 10S and 19S units rather than distinct species. The fraction of material present as 19S dimer estimated from sedimentation velocity agrees with the fraction determined by light scattering.

Glandular cells, possibly osmoregulatory, in the gill leaflets of Callinectes. EUGENE COPELAND.

The respiratory platelets of the gill of *Callinectes* have a cellular patch near the efferent vessel that blackens with both silver nitrate and with osmic acid. The patch is small in sea water-adapted animals and increases in area several-fold in fresh water-adapted animals. Sea water animals forced to adapt to fresh water (ca. 10 days) show marked hypertrophy, not only in size of the patch but also in thickness of the cells concerned. A branching network of fine vessels, limited to the patch, converge in the area of the efferent vessel. The specific cells are single-layered and highly organized. The cell surface adjacent to the gill cuticle is indented by many parallel tubules that seem to have pynocytosis at the ends. This adjoins a region populated by Golgi figures and rough endoplasmic reticulum. This in turn gives way to a region (about $\frac{2}{3}$ of the cell) composed of numerous, long mitochondria arrayed parallel to each other and in the axis of the cell. A network of smooth endoplasmic reticulum surrounds the mitochondria and empties to the plasma surface of the cell. The inter-cell membranes show two types of folds. One is an even, widely spaced fold that has vacular-like spaces trapped between the double membranes which at times show fine periodic cross-connections. The other type is an interdigitating, parallel folding that may have mitochondria packed in the crevices, much as seen in the kidney tubule. It is hypothesized that the cells may account for the absorption of salts by the gills when the animal adapts to fresh water.

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Two kinds of potentials in the dinoflagellate Noctiluca miliaris. ROGER ECKERT AND CONSTANCE QUINN.

The luminescent flash of *Noctiluca* is invariably preceded by an all-or-none negative-going action potential as recorded by conventional glass microelectrodes from the large vacuole. This potential is seen only in response to a stimulus (*e.g.*, electrical or mechanical), and ranges in magnitude up to 70 mV, developing at a maximum rate of 10–25 volts per second.

This "fast" potential is also obtained by passing inward (anodal) current through a second electrode in the vacuole. The fast potential takes off from internally negative potential displacements of 100–150 mV. Since this represents the IR drop across both the external and the vacuolar membranes, the actual voltage threshold for the fast potential is not known.

A different potential is seen temporally related to the spontaneous tentacle movements. This potential is somewhat more variable in shape, is not as strictly all-or-none, and is of a longer time course than the fast potential. It is, however, of the same polarity. The "slow" potentials have a maximum rate of development of about 5 volts per second, and are typically of smaller magnitude than the fast potentials.

Slow potentials can be evoked by outward (cathodal) current pulses, opposite in polarity to that which evokes the fast potential. If the internal potential is positively displaced in this manner up to 20–40 mV, there is an active additional increase in positivity lasting several hundred milliseconds after the termination of the current pulse. This is followed by the negative-going slow potential. The latency of the slow potential is reduced as the current pulse amplitude is increased.

The slow potential is inhibited by closely preceding fast potentials. The two potentials do not add completely, but partial addition seems to occur. It is suggested that the two potentials represent two active membranes.

A preliminary study of thromboplastin generation in the smooth dogfish, Mustelus canis. Rosemary W. Eskridge, Frank A. Belamarich and Douglas M. Surgenor.

Thromboplastin generation occurs in mammals when plasma (adsorbed by barium sulfate), serum, platelets and calcium are incubated in a saline medium and buffered at pH 7.3. *In vitro* coagulation in the smooth dogfish is a very gradual process, often requiring more than 24 hours. Clotting takes place in less than 7 minutes, however, after the addition of either 40–70 m*M* calcium, skin extract, or celite. Serum from the clots formed when no addition is made, or in the presence of celite, promotes the generation of thromboplastin in the above system, while serum from clots formed in the presence of skin extract or high calcium appears to be inhibitory.

Using a homologous system, in which all blood fractions are obtained from the dogfish, thromboplastin is formed slowly at physiological calcium levels of 8 m*M*, but generation is rapid at 40 m*M* calcium. Thromboplastin is not formed in the absence of serum or adsorbed plasma and is formed only slowly in the absence of cells. Fractionation of the serum by adsorption onto barium sulfate, followed by adsorption onto celite, and using the two eluates indicates the presence of at least two active factors in the serum. Both red and white cells promote thromboplastin generation, activity residing in the cell ghosts and the cytoplasm. Most cytoplasmic activity is sedimented at 20,000 *g*. The remaining cytoplasm from white cells has some activity, while that from red cells is inhibitory. Sedimentation of the red cell cytoplasmic supernatant solution at 80,000 *g* yielded a pellet with slight activity, the final supernatant solution being inhibitory.

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The bacterial luciferase system: reactions with hydroxylamine. Linda Garrick, Deric Bownds, Stephen Harrison, Richard Muesing, James Spudich and J. W. Hastings.

Purified luciferase from *Achromobacter fischeri* reacts sequentially with $FMNH_2$, oxygen, and a long-chain aldehyde. When no aldehyde is added, only a very small amount of light is emitted. At the same time it can be shown that the enzyme reacts normally with $FMNH_2$ and oxygen to produce an intermediate fully capable of reacting with aldehyde. These experiments were carried out to determine whether the low level of light which comes from this intermediate when aldehyde is not added is caused by a small amount of contaminating aldehyde or whether the "dark" reaction whereby the intermediate disappears is, in fact, a low quantum yield reaction.

Hydroxylamine, which reacts with aldehydes through the formation of oximes, was incubated with enzyme before initiating the reaction. With 10^{-3} M hydroxylamine the amount of light emitted was markedly reduced, indicating that contaminating aldehyde had been present. The quantum yield compared with that obtained with aldehyde was decreased by a factor of 1000. Under these conditions it could again be demonstrated that the first two reaction steps occur normally and that a full amount of intermediate capable of reacting with aldehyde was present.

The residual luminescence appeared resistant to reaction with higher concentrations of hydroxylamine (up to 0.3 M) and could not be removed by dialysis with buffer or hydroxylamine. It is concluded that a light-emitting reaction, presumably having a very low quantum yield, does occur in the absence of long-chain aldehyde.

A transfer RNA from Griffithsia globulifera. HARRY M. GOUGH AND MAIMON NASATIR.

In the synthesis of proteins, transfer RNA (tRNA) has the function of carrying an amino acid to a ribosome where the amino acid is polymerized into a polypeptide. During an investigation of the nucleic acids of the red alga *Griffithsia globulifera,* an RNA fraction was isolated which behaved as tRNA in the cell-free amino acid incorporating system described by Nirenberg and Matthaei.

G. globulifera was gathered in Little Harbor. The plants were killed by plunging them into −10° C. acetone, dried by lyophilization, ground into a fine powder, and de-pigmented by usual methods. The gray powder which remained was extracted three times with sodium lauryl sulfate (5%) ; the extracts were combined and precipitated with ethanol. The precipitate was extracted with 0.5 M NaCl and deproteinized by shaking with isoamylalcohol-chloroform. The aqueous layer was removed, precipitated with ethanol and taken up in 0.5 M NaCl. This salt extract was centrifuged at 32,000 g for one hour. The pellet was discarded. This salt extract was fractionated on a Sephadex G-100 column.

An RNA fraction having the molecular weight of slightly less than 100,000 was isolated. This RNA was used in the polyuriadylic acid-primed synthesis of polyphenylalanine. Controls run with no tRNA were also done. These experiments showed a 2.3- to 6-fold increase in the radioactive component rendered insoluble in "hot" acid, compared to the controls. Using the optical density at 260 mμ as an estinmate of the amount of tRNA present, the *G. globulifera* tRNA is as efficient in this system as tRNA prepared from *Escherichia coli.*

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Thermodynamics of G–ADP actin polymerization. ROBERT J. GRANT, S.J.

It was reported in these abstracts last year that G-ADP actin polymerization is a temperature-dependent process, that is, in the presence of salt it will polymerize as the temperature is raised and depolymerize reversibly as the temperature is lowered.

That this process is a true thermodynamically reversible reaction is shown by the fact that $G \rightleftharpoons F$ equilibrium states can be maintained at various temperatures. These are measured by following the viscosity as the temperature of the actin solution is slowly raised and then slowly lowered. That these states of intermediate polymerization are true equilibrium states is supported by the following: the curves of viscosity vs. temperature for both the polymerization and depolymerization reactions follow the same path.

From these equilibrium values the thermodynamic parameters have been estimated and support the assumption that G-ADP actin polymerization is an entropy-driven reaction.

In the presence of orthophosphate or in the presence of tropomyosin the actin becomes "locked" in the polymerized form. These effects may suggest how the actin filament is stabilized in the muscle.

In an attempt to establish the involvement of water in the polymerization reaction, the effect of tetra-butyl ammonium ion was investigated. In low concentrations, 2–4 mM, it causes a rapid depolymerization at an otherwise polymerizing temperature. The effect of deuterium oxide is being investigated.

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Thyroid hormone-induced swelling of isolated dogfish liver mitochondria. ROGER L. GREIF AND JOSEPHINE A. ALFANO.

Suspensions of mitochondria isolated in 0.25 *M* sucrose from livers of smooth dogfish, *Mustelus canis,* will swell if incubated with thyroid hormones. Less protein is released into the suspending medium during incubation of mitochondria from dogfish liver than from rat liver, and in the former species the amount of released protein does not increase during swelling. The sensitivity to added hormone is increased by isolating dogfish mitochondria in 0.44 *M* sucrose and incubating at 30° C. in 0.15 *M* KCl buffered to pH 7.6. The most active swelling agent tested is 3,5,3′-triiodothyroacetic acid, with effects detectable at 10^{-6} *M*. At 5×10^{-5} *M* concentration, 3,5,3′-triiodo-L-thyronine is more effective than L-thyroxine or 3,3′,5′-triiodo-D,L-thyronine. Barely detectable effects are produced by 3,3′-diiodo-D,L-thyronine, and 3-iodo-D,L-thyronine and 3,5-diiodo-L-tyrosine do not influence mitochondrial swelling. The swelling can be prevented by 5×10^{-3} *M* ATP.

The *in vitro* effects upon dogfish liver mitochondria of compounds related to thyroxine correlate better with *in vivo* effects upon amphibian metamorphosis than with *in vivo* calorigenic effects upon rats. Since the adult dogfish does not appear to show a calorigenic response to thyroid hormones, the ability of the biologically active thyronines to cause mitochondrial swelling may be related to other hormone effects upon fishes.

This investigation was supported by grants from the U. S. Public Health Service, National Institutes of Health, and from the National Science Foundation.

Effect of acclimation to various salinities on potentials in isolated crab gills. LINDA B. HABAS AND C. LADD PROSSER.

Callinectes sapidus, a euryhaline crab, maintains blood hyperosmotic to dilute media. Isolated gills of *Callinectes* perfused with sea water show a positive potential inside the afferent vessel of +3–4 mv with respect to a sea water medium. This potential decreases to zero in 80% sea water and becomes negative in more dilute media, reaching −6 mv in 50% sea water. In higher salinities, the potential becomes more positive, reaching +6 mv in 150% sea water. Replacement of chloride by propionate makes no difference in the magnitude of the potential, while replacement of sodium by choline gives rise to a large negative potential. The point of zero potential can be changed by acclimation of the crab. Animals acclimated to 120% sea water show a negative gill potential below 140% sea water and those acclimated to 110% sea water show a negative potential below 130% sea water. Crabs kept at 3% sea water for two weeks show a positive potential down to 50% sea water. Preliminary experiments with Na^{24} show that efflux of sodium from the gill is rapid, with a half-time of 15–20 minutes. It is postulated that the negative gill potential results from passive loss of sodium, while the positive potential is due to presumably active uptake of sodium exceeding the passive loss. Acclimation is thought to increase the active transport capacity, enabling the gill to maintain a positive potential in more dilute sea water. In isolated gills of *Libinia emarginata,* a stenohaline, osmoconforming crab, there are no potentials at all in external media of 50–100% sea water.

The loss of osmotic response during squid sperm maturation. SHIRLEY HILDEN AND HIDEMI SATO.

The sperm nucleus of the squid, *Loligo pealii,* gradually condenses laterally during the process of maturation but does not change its length. The sperm nucleus shows a ten-fold decrease in volume and a change in birefringence from a retardation of almost zero to thirty millimicra. The width of the mature sperm is 1.45 micra; therefore the coefficient of birefringence is 0.02. This coefficient of birefringence, which is negative in sign, is characteristic of DNA. Judging from the gradual increase of negative birefringence of the rod-shaped squid sperm nucleus throughout spermiogenesis, the DNA molecules which compose the sperm chromosomes apparently become tightly packed and aligned in the mature sperm.

During maturation, the responses to osmotic changes decrease gradually, as shown by volume changes in hypotonic solutions of artificial sea water and sucrose solutions. The mature sperm shows no volume change even in distilled water. In spite of the large swelling in hypotonic solutions, the immature sperm nucleus loses only a small amount of its total retardation. This probably reflects an increase in distance between the DNA molecules without their disorientation. Experiments using D_2O indicate that all stages are permeable to water.

These experiments have indicated changes in osmotic response during spermiogenesis which produce an extreme resistance of the squid sperm to conditions normally adverse to a cell. These changes cannot be explained in terms of changes in membrane permeability to water.

Disappearance of colloidal and diffusible solutes from tissue spaces in Loligo and Limulus. CHESTER HYMAN.

In most mammalian tissues, locally injected diffusible solutes disappear at rates that vary directly with blood flow in the exchange vessels in the area. Similarly injected colloids disappear at considerably slower rates and are thought to be removed exclusively by lymphatic drainage. For example Na^+ disappears from sub-cutaneous tissues in mammals with a half-time of about 8 minutes while half-time for removal of labelled albumin is at least 8 times as long. The ratio of these indices of local blood and lymph circulations may be considered a measure of the "openess" of the circulation, *i.e.*, the integrity of the blood-parenchymal barrier.

Simultaneous injections of radioactive Na^+- and I^{131}-labelled colloid (human serum albumin or *Limulus* blood) were made into the muscle of the first tarsus of the fourth walking leg of a horseshoe crab (*Limulus polyphemus*) or the distal portion of a tentacle of a squid (*Loligo pealei*). At measured intervals the amount of label remaining at the injection site was determined by placing the member into a plastic test tube in a well scintillation counter, with appropriate shielding from the remainder of the animal. Precautions were taken to minimize bleeding, to maintain constant geometry during successive counts, to permit the animal normal free movement between determinations and, in the case of the squid, to keep the animal in properly aerated sea-water at all times—even during the counting.

In 8 experiments on *Limulus* the disappearance rates (half-times) for Na^+ and for colloids averaged 124 and 156 minutes, respectively. In 5 experiments on *Loligo* the corresponding averages were 26 minutes for Na^+ and 105 minutes for iodinated *Limulus* blood. The relatively rapid Na^+ clearance from squid tissue suggests a high degree of vascularity. The ratio of half-times for diffusible:colloidal solute removal was 1.26:1 for *Limulus* and 4.05:1 for *Loligo*. These values are consistent with the accepted notions concerning the openess of the circulation in these two forms.

Heavy water enhancement of mitotic spindle birefringence. SHINYA INOUÉ, HIDEMI SATO AND ROBERT W. TUCKER.

When developing eggs of the sea urchin *Lytechinus variegatus* were placed in sea water made up in heavy water (D_2O) and H_2O, the cells in mitosis but not cleavage were reversibly arrested at D_2O concentrations of 45% and higher. In sea water containing 30% to 40% D_2O the birefringence of the spindle fibers rose above normal but mitosis proceeded. When sea water with 45% to 60% D_2O was applied during mitosis, the spindle birefringence rose higher and then fell; mitosis was arrested unless the cell was exposed to D_2O during or after anaphase. At these concentrations, the effect of raising the spindle birefringence is a sensitive function of the exact stage of mitosis at which the D_2O is applied. At 50% D_2O the retardation can be doubled and may reach a maximum of 7 $m\mu$ only if D_2O reaches the spindle at a particular 10-second interval in anaphase. This differential sensitivity of the spindle fiber birefringence to D_2O at different stages of mitosis closely parallels its sensitivity to temperature. The birefringence increases at elevated temperatures and vanishes reversibly at lower temperatures. The sensitivity rises once during spindle formation, becoming minimum at metaphase, then rises to a higher maximum again in anaphase and gradually decreases towards telophase. The rise of spindle fiber birefringence in D_2O adds to the list of agents which similarly affect (1) the spindle fiber birefringence, (2) the "gel strength" of cleaving sea urchin eggs, and (3) the polymerization of tobacco mosaic virus S proteins. This fact increases the likelihood that a common mechanism underlies these various processes.

Lysosomal enzymes in leucocytes of Mercenaria mercenaria and Asterias forbesi. AARON JANOFF AND EUGENIA HAWRYLKO.

Rabbit polymorphonuclear leucocytes and macrophages contain many of the lysosomal acid-hydrolases described in rat liver cells by de Duve. In leucocytes of mammals, these enzymes are also localized within specific cytoplasmic granules (lysosomes) and are only released during phagocytosis, whereupon they pass into the phagocytic vacuoles concerned with digestion and detoxication of engulfed materials. Amoeboid leucocytes of two invertebrates (quahog and common starfish) were examined for the presence of several of the acid-hydrolases known to occur in rabbit phagocytic cells. Slight acid-phenolphthalein-phosphatase activity and considerable acid-RNAase activity were found in leucocytes of both species. Appreciable beta-glucuronidase activity was also present in the quahog cells, but this enzyme was not detected in leucocytes of the starfish. By far the highest activity of the three enzymes assayed was that of RNAase. Differential centrifugation of cell-lysates was carried out and enzyme activity was assayed upon the following four cell fractions: nuclei, lysosomes (plus mitochondria), microsomes, and cell-sap. In general, the activity of each enzyme in both species was as high or higher in the cell-sap as in the lysosome fraction. This pattern differs markedly from that of the mammalian leucocyte in which enzyme activity is largely restricted to the granules in resting cells. Preparation of subcellular fractions in different molarities of sucrose and by different cell-lysis techniques had essentially no effect on the resultant enzyme distribution pattern. It is not known whether this atypical enzyme localization represents the normal condition in invertebrate leucocytes or results from continuous phagocytic activity on the part of these cells *in vivo*.

Comparative aspects of the "renin-angiotensin" system. GABOR KALEY, G. ALAN ROBISON AND BARBARA LUBBEN.

In view of the recently suggested role of angiotensin as a hormonal regulator of aldosterone secretion in mammals, in addition to its role in blood pressure regulation, it was of interest to investigate the presence or absence, and possible significance of this polypeptide in a variety of lower forms. Aquatic animals might be expected to differ widely in their abilities to produce angiotensin and consequently in their dependence on aldosterone for the maintenance of their internal environment. In mammals, angiotensin is known to be produced by the action of a nephrogenic enzyme, renin, on its substrate, angiotensinogen, a component of the alpha-globulin fraction of plasma. Renin was assayed by the *in vivo* injection of purified kidney extracts into the rat and the dog, and also by the *in vitro* production of angiotensin after incubation of kidney extracts with rat, dog and, whenever possible, homologous plasma. Results indicate that, contrary to previous reports, kidneys of *Carassius* (carp) and *Ameiurus* (catfish), both fresh-water teleosts, do not contain renin. Similarly, no renin activity was demonstrable in kidneys of two marine fish, *Opsanus tau* (toadfish) and *Mustelus* (dogfish). In contrast, however, kidneys of *Paralichthys* (flounder) were found to have amounts of renin comparable to those found in mammalians. Extracts of kidneys of *Delphinus* (dolphin) possessed renin activity which approximated that found in rat kidney. In mammals, renin is localized in the granular juxtaglomerular cells. Histologic examination of kidneys of all animals studied indicated that presence of granular juxtaglomerular cells was consistently parallelled with renin activity.

Experiments leading to a new interpretation of the mechanism of mitotic apparatus isolation. ROBERT E. KANE.

Several years ago the author developed a method for the direct isolation of the mitotic apparatus or MA (spindle, chromosomes and asters) from dividing echinoderm eggs, using a 1 M solution of hexanediol or other long chain glycol, buffered at pH 6.2 to 6.4 as isolation medium. This method gave high yields of clean MA, but the role of the glycol in isolation was not known.

Investigations have been carried out to determine whether the function of the glycol is unique, or whether other compounds, such as alcohols, could be substituted for it with a suitable change in procedure. A 1 M solution of ethanol is ineffectual in isolation at the pH values used with the glycols, but gives satisfactory isolates at lower pH values. The reverse also holds, that is, isolation is possible in ethanol at higher pH values if the concentration is increased. In particular, the concentration of ethanol required for isolation at pH 6.4 is similar to that required for hexanediol if both are expressed on a per cent rather than a molar basis. This relation has been found to hold for a number of alcohols and glycols, indicating that osmotic considerations are of little importance and that a dehydration of the MA is the fundamental process involved.

The concentration of alcohol or glycol required for isolation depends directly on the pH, rising from 0 to 22% in the pH range from 5.5 to 8.0.

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Studies on the survival of dark and bright mutants of luminescent bacteria in sea water. ALEX KEYNAN, CAROLYN VEEDER AND J. W. HASTINGS.

The functional importance and possible survival value of light emission by marine bacteria has been a subject of considerable interest. An experimental approach to an investigation of this question was suggested in these meetings two years ago. We reported that in a complete medium the dark mutants grow faster than the parent luminescent bacterium. This seemed to be explainable, since the dark mutant presumably has a greater energy supply available for growth. However, since luminous bacteria are abundant in nature, even though dark mutants occur frequently, it was concluded that in nature there must exist conditions under which there occurs a positive selection for luminescence. Since the normal dispersal of these bacteria presumably occurs in sea water, where little or no growth takes place, the mere capability of cells to survive in sea water might be of significance. When mixtures of dark and bright mutants of luminous bacteria were diluted into autoclaved sea water, the death rate of the darks exceeded that of the brights by a factor of at least three. This observation was repeated with the dark and bright mutants of two completely different strains. Although this fact strongly suggests it, it remains to be proved that the light-emitting system in itself is responsible for the difference in survival in sea water under non-growing conditions. One could imagine that the light-emitting system could have survival value if it is linked with the ability to get rid of excess "electron pressure" in a cell just washed from a rich medium into the sea. Indeed, it has frequently been suggested that luminescence functions to drain off "excess energy" in cells, but to our knowledge, no one has experimentally demonstrated conditions where it might be advantageous to do so. In the present case, where unbalanced metabolism might readily occur upon dilution into sea water, the rapid release of excess energy might be of survival value.

Further studies on an antibacterial factor in the blood of Phascolosoma gouldii. STUART M. KRASSNER.

Whole blood from *Phascolosoma gouldii* was tested for antibacterial activity against a slow growing chromogenic *Vibrio* isolated from *Limulus polyphemus* by Dr. F. B. Bang. The test system consisted of 9.4 ml. of sea water nutrient broth, 0.1 ml. of a 10^6 dilution of a 12–24 hour broth culture and 0.5 ml. of *P. gouldii* blood. Control tubes contained broth and bacteria without blood or with heat-inactivated blood. Most tests were performed at 11° C., a temperature at which the antibacterial activity was stable. Samples were withdrawn after 24 and 48 hours of culture and added to melted ZoBell's sea water agar cooled to about 42° C., which was then poured into Petri dishes. These were incubated at room temperature. Heating the blood for 10 minutes at 70° C. or allowing it to stand for 24 hours at room temperature destroyed the antibacterial activity. Freezing and thawing the blood also destroyed the activity. Heated or frozen blood showed growth promoting activity. Dialysis of the blood in Visking tubing against sea water at 4° C. caused some loss of activity. Addition of the dialysin to the dialyzed blood did not restore normal titer of antibacterial activity. Antibacterial activity was found in both the cellular and fluid portion of the blood but was stronger in the latter. In tests on a *Gaffkya* lobster pathogen supplied by Dr. H. Rabin, it was found that growth was inhibited by *P. gouldii* blood.

Studies on the dogfish lens: (a) membrane permeability, (b) albuminoid RNA. SIDNEY LERMAN, SEYMOUR ZIGMAN AND Y'HESKEL A. SA'AT.

(a) Recent studies indicate that there is an active transport of ions and amino acids into the mammalian lens. This process appears to be located along the anterior surface of the lens (which contains the epithelial cells) while the movement of substances across the posterior lens capsule occurs by means of simple diffusion. Studies on the isolated capsules of the dogfish lens (immersed in elasmobranch Ringer's medium) were performed with C^{14} uracil and leucine. The rates of penetration of these substances through the posterior lens capsule (which lacks the epithelial layer) were twice those obtained with the anterior lens capsule (which contains a contiguous layer of epithelium). When the temperature was lowered to 5° C. or when ouabain or cyanide was added to the outside medium, there was no significant change in the rate of penetration of the test substances through the posterior capsule. However, there was a significant increase in the rate of penetration of these substances through the anterior lens capsule.

(b) The lens contains four types of RNA; these include soluble RNA, microsomal RNA, messenger RNA, and albuminoid RNA. This latter fraction is closely associated with the albuminoid (or insoluble) lens protein located mainly in the relatively acellular core of the lens. It is metabolically active as indicated by *in vivo* and *in vitro* P^{32} and C^{14} uracil incorporation studies. Its base composition is essentially similar to that of the microsomal RNA fraction. It displays an aging effect similar to the changes which occur with albuminoid protein; that is, a marked increase in the relative concentration of albuminoid RNA as the lens ages. In the young dogfish (pup) albuminoid RNA comprises approximately 4–5% of total lenticular RNA; in the mature fish this rises to a value of 25%.

The role of Gram negative endotoxin in the extracellular coagulation of Limulus blood. JACK LEVIN AND FREDERIK B. BANG.

Endotoxin of a *Vibrio* species, pathogenic for *Limulus,* causes the liquid phase (pre-gel), obtained when whole *Limulus* blood clots, to form a solid gel. This gel does not re-gel when agitated and fractured, and does not cause additional pre-gel to gel. This reaction appears to be similar to the conversion of fibrinogen to fibrin, although the nature of the clottable protein in *Limulus* is unknown.

Plasma, containing no cellular material, did not gel when endotoxin was added. Agitation of whole blood, which increased the disruption of amoebocytes, increased the rate of release of material necessary for the reaction with endotoxin.

There was a direct relationship between endotoxin concentration over a million-fold range and the time of onset of gelation, but the final degree of gelation was not affected. This reaction was not inhibited by sodium citrate or heparin, in final concentrations of 3% and 1000 U/ml., respectively; but EDTA (0.6%) prevented it. This inhibition was not reversed by replacement of calcium and magnesium ions, alone or in combination ($M/5$ to $M/800$). Samples taken from a mixture of pre-gel and endotoxin caused fresh pre-gel to clot, presumably due to residual endotoxin. Once final gelation had occurred, this capability was lost. Cell-free plasma was not affected.

Limulus muscle extract was inactive, while a commercial preparation of bovine thrombin clotted pre-gel. This activity was lost when the thrombin preparation was heat-inactivated.

The kinetics of the reaction suggest that an enzyme system is responsible for the conversion of pre-gel to gel.

Deoxyribonucleases of Mustelus canis liver. Lawrence Levine and Helen Van Vunakis.

Deoxyribonuclease activity toward native and thermally denatured T_4 bacteriophage DNA has been determined by immunochemical procedures in homogenates of various tissues from different species. Whereas homogenates of kidney, heart and brain of the smooth dogfish (*Mustelus canis*) have nucleases which attack native and denatured DNA to equal extents, the liver homogenates have 15 to 30 times more activity toward denatured DNA. The ratio of activities toward denatured and native DNA in liver homogenates from one spiny dogfish (*Squalus acanthias*), two northern sting rays (*Dasyatis centroura*), one dusky shark (*Carcharhinus obscurus*) and one blue shark (*Prionace glauca*) were 1:1, 1:3, 3:1 and 8:1, respectively. The nuclease activities of the smooth dogfish liver were partially characterized, using an immunological assay with native and denatured DNA as substrates. The enzyme hydrolyzing denatured DNA is inhibited by p-hydroxymercuribenzoate; its activity is enhanced by Mg^{++} and 2-mercaptoethanol and is optimal at 0.1 *M* NaCl. The enzyme attacking native DNA is not inhibited by p-hydroxymercuribenzoate; its activity is enhanced by Ca^{++} as well as Mg^{++} and is optimal at 0.015 *M* NaCl. Both nucleases are relatively heat-stable, losing 50% of their activity in one hour at 54°. The nuclease digesting denatured DNA has greater activity at 37° than at 15°. It passes rapidly through Sephadex 200; it is absorbed on DEAE cellulose at low ionic strength at pH 7.2 and can be eluted with 0.2 *M* buffer. It is precipitated by 50% acetone and between 30% to 50% ammonium sulfate. Its rate of hydrolysis suggests that digestion proceeds endonucleolytically.

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Uricase instability without simultaneous change in activity caused by exposure to urea. Lu-Ku Li, Aurin M. Chase and Selma L. Lapedes.

It has been reported (Chase, *Biol. Bull.,* 1956, 1957) that uricase is both reversibly inhibited and irreversibly inactivated by urea, the sensitivity of the enzyme being greater in borate than in phosphate or glycine buffers.

Recent experiments involving the action of urea on Worthington's purified uricase suspension in 0.1 *M* glycine buffers of pH's 9.0 to 9.3, at 26° C., indicate that the stability of the enzyme can be decreased without an immediately accompanying change in its catalytic action. Activities were measured spectrophotometrically (Beckman DU and Cary, Model 14) by recording the initial rate of disappearance of the absorption band of uric acid at 292 mμ.

When this purified uricase was allowed to stand in glycine buffer containing 0.6 *M* urea and aliquots were periodically assayed, the activity decreased only slightly (and no more than did that of the controls) over a nine-hour interval. If allowed to stand in 6.0 *M* urea in the same buffer and aliquots of *this* mixture were periodically diluted to reduce the urea concentration to 0.6 *M* and the enzyme activity was immediately measured, essentially the same activities were observed as when the enzyme had been exposed *throughout* to 0.6 *M* urea. When, however, the enzyme *remained* in 0.6 *M* urea after previous exposure for various times to 6.0 *M* urea, its activity fell rather rapidly. Two independent series of experiments with different lots of purified uricase yielded entirely similar results.

It would appear that uricase may be altered structurally in 6.0 *M* urea so that, although its activity can be recovered on dilution of the urea to 0.6 *M,* its *stability* has been greatly impaired.

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Uterine response to 5-hydroxytryptamine in the clasper-siphon secretion of the spiny dogfish, Squalus acanthias. T. Mann and L. C. Prosser.

The male copulatory organ (clasper) of the spiny dogfish, by means of which semen passes at copulation to the female, communicates with a male accessory organ, the clasper siphon, which is an extremely rich source of 5-hydroxytryptamine (serotonin). A pair of siphons contains on an average 0.3% serotonin, that is, up to 20 mg. or more, per animal (Mann, 1960).

Among the known pharmacological effects of serotonin on mammalian organs is the ability to induce contractions of the uterus. We have demonstrated a similar effect using isolated uterine horns of the spiny dogfish (animals caught in July–August; about three months pregnant; conceptus removed), 10–20 cm. long, suspended in 125 ml. aerated medium (%: NaCl 1.64, KCl 0.09, $CaCl_2 \cdot 2H_2O$ 0.15, $NaHCO_3$ 0.04, NaH_2PO_4 0.007, glucose 0.1, and urea 2.2). Uterine horns which did not show spontaneous contractions responded upon addition of 50–500 μg. serotonin with powerful brief contractions (10–20 second contraction time; 20–60 second half-relaxation time); these were often followed by smaller periodically occurring movements, occasionally persisting for several hours, even when serotonin had been washed out with fresh medium. However, when contracting spontaneously or after induction with either serotonin or acetylcholine, the dogfish uterus appeared to be relatively insensitive to a further dose of serotonin. When the siphon sacs of spiny dogfish were flushed out with 10 ml. water, and 0.1–0.2 ml. of such fluid (representing 0.2–0.4 mg. serotonin) was applied to the isolated uterus, powerful contractions were recorded, similar to those observed in response to a solution of serotonin creatinine sulphate; these were followed by the typical recurrent movements. By eliciting uterine contractions, the clasper-siphon secretion may well play a physiological role during copulation, perhaps by facilitating sperm ascent to the site of fertilization.

A preliminary report of studies with natural hemagglutinins from Golfingia gouldii and Limulus polyphemus. JOHN J. MARCHALONIS.

In an exploratory study of the natural serological properties of invertebrate body fluids, the ability of coelomic fluid or blood to agglutinate vertebrate erythrocytes was examined by means of test tube dilutions. Serum prepared from the coelomic fluid of *Golfingia gouldii* possessed a low-titer, heat-labile agglutinin which clumped sheep, chicken, and rabbit red blood corpuscles. *Limulus* serum, likewise, contained a heat-labile material which agglutinated the above vertebrate erythrocytes. However, this hemagglutinin was detectable at much higher dilutions. Although normal *Limulus* serum agglutinated the three vertebrate red blood cells to approximately the same titer, *Limulus* serum which was absorbed with sheep erythrocytes lost most of its ability to agglutinate sheep cells, but retained a relatively large capacity to clump the other test erythrocytes.

External fertilization of Botryllus schlosseri eggs. ROGER MILKMAN AND MARTHA BORGMANN.

Eggs of the compound ascidian, *Botryllus schlosseri,* have been removed and fertilized externally. This procedure, which has not been achieved before, is of interest in view of the unusual promise of this species for genetical research.

In the fairly well synchronized sexual and budding cycles, zooids of one generation are resorbed and replaced by those of the next. At this time, eggs leave pouches on the sides of the new zooids and enter the atrial cavity. After a few hours the germinal vesicle breaks down, and only then is fertilization possible. About this time, the new zooids' siphons open, admitting exogenous sperm. Isolation of the colony just before the new siphons open will usually prevent fertilization until the new testes mature in about two days, at which point selfing can occur, but colonies vary as to the time of resorption of the old zooids, and late resorption can permit sperms from the old generation to fertilize the new eggs. Fertilized eggs develop into larvae which are extruded during zooid resorption, or, if immature then, from the common atrial cavity when mature.

Unfertilized eggs are easily removed and fertilized, using crushed mature testes or, better, placing them with another colony. Sperms are apparently released intermittently from testis maturation to resorption. The percentage of successful fertilizations is presently only about 30%; some eggs are still fertilizable 36 hours after removal; early cleavage stages have previously been carried through metamorphosis.

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Chemotaxis in Campanularia sperm: species specificity. RICHARD L. MILLER.

After the phenomenon of chemotaxis in the sperm of the hydroid *Campanularia flexuosa* was discovered in 1962, it was thought desirable to look for chemotaxis in a second species of *Campanularia* and see if the substances involved were species-specific. The species *C. calceolifera* was used. Experiments were set up so that a female gonangium of both species was present in a suspension of sperm from only one species. In all cases, the sperm was attracted to and clustered about the tip of the *homologous* gonangium. However, a side reaction was observed between *C. flexuosa* sperm and the female gonangium of *C. calceolifera*. The attraction here was temporary and did not show the characteristic intense clustering at the opening of the gonangium of *C. calceolifera* when *C. calceolifera* sperm was used. No side reaction was observed in the case of *C. calceolifera* sperm vs. the *C. flexuosa* gonangium. All attempts to extract the chemotactant from *C. flexuosa* gonangia have so far failed.

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Intestinal absorption of D-glucose in various species of fish. X. J. MUSACCHIA AND R. A. HINES.

The question of active transport in fish intestine has not been fully answered. *In vitro* intestinal sac preparations from scup, *Stenotomus versicolor,* and catfish, *Ictalurus nebulosus,* have been used and tenuously credited with demonstrating characteristics of active transport of D-glucose.

Experiments with intestine from catfish, scup, and puffer, *Spheroides maculatus,* were conducted with a view toward providing additional information concerning absorption phenomena. Using D-glucose, 10 mg.%, intestinal segments (upper, middle, and lower) from six catfish showed levels of mucosal uptake in micromoles/gm. dry wt./30 min. ranging from 5.19 to 21.83 and serosal increase from 8.44 to 16.74.

Aiming toward further refinement of methods for studies with marine fish intestinal preparations, two series of experiments were conducted: (1) varying time of incubation; (2) varying temperature of incubation. Using scup intestine, best results were obtained at 30-minute and 45-minute incubations. There was, in micromoles D-glucose/gm. dry weight, mucosal uptake from 9.61 to 19.13 and serosal increase from 1.93 to 6.25. When the preparations were incubated for 60 minutes, results were often erratic, *i.e.,* sugar was emitted into the mucosal fluid or absorbed from the serosal fluid. Incubations of scup intestinal segments for 30 minutes at 23° C., 26° C., and 27° C. resulted in uni-directional change, *i.e.,* there was mucosal uptake and serosal increase of D-glucose. At 29° C. mucosal uptake was not constant and at 30° C. D-glucose was emitted from both mucosa and serosa.

Using 12 puffers, four areas of intestine were examined. Mid-intestine showed greatest levels of D-glucose mucosal uptake from 9.23 to 24.00 and serosal increase from 1.93 to 5.76, in micromoles glucose/gm. dry wt./30 minutes. Anaerobic conditions (100% nitrogen) inhibited mucosal uptake and serosal increase of the sugar.

Preliminary experiments with uneverted intestinal sacs from lungfish, *Lepidosiren* sp., showed absorption of D-glucose from mucosal fluid and increased concentrations on the serosal side.

A DNAse from Griffithsia globulifera. MAIMON NASATIR AND HARRY M. GOUGH.

While studying the nucleic acids of the red alga, *Griffithsia globulifera,* difficulties were encountered in preparing DNA of high molecular weight, even at pH's above 7 and in the presence of 0.05 *M* sodium citrate. This led us to look for a DNAse the properties of which differed from both DNAse I and DNAse II.

An acetone powder of *G. globulifera* was prepared from plants which were collected in Little Harbor. This powder was homogenized in cold 0.5 *M* NaCl and centrifuged at 32,000 *g*. After centrifugation, the supernatant fraction was dialyzed in the cold against three changes of distilled water for 18 hours. This crude preparation had DNAse activity when highly polymerized calf thymus DNA was used as the substrate.

The rate of this reaction was not altered in the presence of 0.1 *M* citrate or by the addition of Mg^{++}. The reaction, however, was stopped by heating the crude preparation for 10 minutes at 100° C. In 0.1 *M* phosphate buffer the crude enzyme preparation had a pH optimum near 7.

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Some properties of Mytilus sperm acetylcholinesterase. LEONARD NELSON.

About 80% of the total sperm acetylcholinesterase activity occurs in the flagellum. Washed sperm, suspended in 0.1 *M* KCl, were subjected to ultrasonic vibration for 45 seconds at 20 Kc. The sperm homogenate was separated by low speed centrifugation; the supernatant, containing tail fragments of varying lengths, was concentrated into a pellet at 10,000 *g* in a refrigerated centrifuge. The Hestrin method was used to measure the hydrolysis of acetylcholine. Optimum enzyme activity occurred in an incubation medium containing 5×10^{-3} *M* acetylcholine chloride, 5×10^{-2} *M* KCl, 5×10^{-2} *M* phosphate buffer and 1×10^{-3} *M* $MgCl_2$. At room temperature, the crude tail enzyme preparation hydrolyzed 125 μg. acetylcholine per mg. of protein per hour while the rate was nearly double that at 45–46° C. Above this temperature, the enzyme was rapidly inactivated. The energy of activation may be calculated to be about 7400 cal./degree/mole. The optimum pH for sperm tail hydrolysis of acetylcholine was at neutrality, measured over a range from pH 5 to pH 9 in acetate, Tris and phosphate buffers. Even though pH 7.4 is given in the literature as the optimum, the tail enzyme exhibited about 40% higher activity at pH 7.0. Intact sperm have about the same specific activity as sonically disrupted or frozen-ground sperm. One may conclude from this, first, that homogenization by these methods does not adversely affect the enzyme and second, that the enzyme is readily accessible to externally supplied substrate. If the enzyme is associated with structures at the surface of the spermatozoon, it may be involved in coordination or propagation of the flagellar wave. That the enzyme is actively engaged in some aspect of the sperm motility was shown by Applegate (1962) who observed that eserine increased the rate of flagellation of live sperm.

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"Circa-tidal" activity rhythms in fiddler crabs. Effect of light intensity. JOHN D. PALMER.

In the study of biological rhythms one finds that rhythms which are 24 hours long in nature have period lengths either slightly longer or shorter than this in laboratory conditions of constant light and temperature. The term *circadian* was coined to describe this phenomenon. Populations of fiddler crabs studied in laboratory constant conditions display the same tidal activity rhythms as they do in nature, *i.e.,* they are active about the time of low tide and inactive at high tide. Underlying this rhythm is a low amplitude solar-day rhythm. In nature, the crabs are not strictly in phase with each other; therefore it is somewhat surprising to find that in the laboratory the rhythm is precisely 24.8 hours long—the length of a lunar day. *A priori,* a "circa-tidal" frequency might be expected. Such precision could be due to the fact that only the average activity of large populations of crabs was considered in the past. Therefore, in the work reported here, single individuals were studied.

Uca pugnax were placed in actographs in a constant temperature room (18° C.). The light intensity could be varied between 0 to 400 ft.c. (with corresponding temperature changes from 18 to 23.5° C.). In an eleven-day study in constant darkness a population of seven crabs displayed a bimodal, activity rhythm with an average period of 25.8 hours. Individuals of this group had periods varying between 25.5 and 25.9 hours. Similar experiments were carried out at 100, 200, and 300 ft.c. In all of these cases the period length of averaged groups did not differ significantly from 24.8 hours. The periods of individuals within this group varied between 24.2 and 25.5 hours. In a nine-day study of a population of seven crabs in 400 ft.c., the average period was found to be 24 hours. All individuals in this group also displayed periods which approximated 24 hours. Under this light intensity (and temperature increase) the lunar rhythm is suppressed and the solar rhythm dominates.

Preliminary report of rhythmic photosynthetic activity in pure cultures of algae. JOHN D. PALMER, LAURA LIVINGSTON, DENNIS ZUSY AND JAMES O'BRIEN.

Pure cultures of *Phaeodactylum tricornutum* and *Gymnodinium nelsoni* were grown in Guillard's Medium "f" under cycles of 12 hours of darkness and 12 hours of illumination. The light intensity during the light period was 600 ft.c. When the cultures approached the stationary phase of growth, 10-ml. samples were withdrawn at the midpoints of the light and dark periods and incubated with 1 μc. $Na_2C^{14}O_3$ for 15 minutes at 600 ft.c. At the end of this time the reaction was stopped, the cells collected on millipore filters, dried, and C^{14} uptake determined. Concurrently, aliquots were extracted with methanol and the chlorophyll content determined spectrophotometrically. Cell counts were made simultaneously.

Using this procedure it was found that the photosynthetic *capacity, i.e.,* the ability of the algae to undergo photosynthesis, was maximal during the light period and minimal during the dark period, the difference being over three-fold. To determine whether the night-time minimal values were a result of the preceding darkness, the cultures were grown in alternating light and dark periods for ten days, and then put into constant light intensities of 0, 20, 40, 80, and 600 ft.c. In constant darkness no rhythm was observed. In 20 ft.c. the rhythm persisted for one cycle. In 40 and 80 ft.c. the rhythm persisted for three days and then damped out. In 600 ft.c. the rhythm persisted for only one cycle. The rhythmic variations in photosynthetic capacity are not caused by concurrent, rhythmic changes in chlorophyll content. The daytime chlorophyll content was actually slightly lower than the night-time value; however, the values were not significantly different.

The data also suggest the presence of a rhythm in cell division. Virtually all cell increases took place during the last half of the dark period and the first half of the light period.

Studies on the antibacterial activity of Golfingia gouldii coelomic fluid. HARVEY RABIN AND FREDERIK B. BANG.

Healthy worms have sterile coelomic fluid. Inoculated bacteria, a marine vibrio, were not recovered five hours after injection. When coelomic fluid from individual worms was mixed with a suspension of 2000 bacteria and incubated for three hours at room temperature, 45% of the fluids inhibited the growth of the bacteria. Inhibition was defined as a 10-fold reduction in colony count compared to bacteria incubated in broth. Inhibition was found 30 minutes after mixing. When incubation was extended to 24 hours, only 30% of the fluids showed inhibition. Thus, with one-third of the active fluids, inhibition decreased on incubation. Bacteria isolated from mixtures in which loss of activity had occurred were susceptible to inhibition. When the coelomic fluid-bacteria mixture was incubated for 24 hours at 11° C., 36 of 39 fluids from fresh uninoculated worms, 8 of 8 from worms inoculated with broth, and 39 of 41 from worms inoculated with vibrio were positive for inhibition. Boiling the coelomic fluid removed activity. Fluids held at room temperature for 10 hours were still active. Of 23 worms positive two days after being brought into the laboratory, 10 were positive 12–15 days later. Of 53 worms, 18 showed activity in the fluid portion of the coelomic fluid after removal of the cells by centrifugation. Various attempts to alter the activity were made by inoculation of the vibrio. On tests of whole fluid at room temperature, there was no difference between 14 worms inoculated with broth and 24 inoculated with vibrio. With tests carried out at 11° C., no difference was found with 8 worms inoculated with broth, 9 uninoculated, and 23 inoculated with vibrio two and 12–15 days before testing. On tests on the fluid portion only, there was no difference between 12 uninoculated worms and 12 inoculated with heat-killed vibrio two days earlier. Results on fluid portions from worms inoculated with live vibrio two days prior to testing showed 6 of 9 inoculated worms and 2 of 8 uninoculated worms positive. Finally, worms inoculated with live vibrio as often as once a day for six days still had active fluids when tested one day after the last inoculation.

Evaluation of an image intensifier tube for microscopic observations. G. T. REYNOLDS, R. D. ALLEN AND S. INOUÉ.

An English Electric Valve Co. transmission secondary emission image intensifier tube with a measured gain of 9×10^5 and a cathode efficiency of *ca.* 0.1 has been evaluated as a possible aid in increasing the speed and/or sensitivity of low light-level microscopic observations, especially with polarized light.

The maximum over-all gain for microscopy, including light losses involved in photographing the phosphor screen output, was *ca.* 10^4. Since the resolution at the phosphor screen is somewhat greater than 20 line pairs/mm., it is in principle possible to resolve distances of the order of 0.5 μ in the microscopic field at a magnification of 100 ×. The proportionality of output brightness at the phosphor screen to input light intensity at the cathode was found to be good over the range of low input intensities at which the contrast discrimination of the eye is very poor. Contrast provided by a bright object in a dark field is faithfully represented by the tube except for thermal and signal-generated noise "leakage" into the dark background. However, the contrast of dark objects in a bright background tends to be spoiled by signal-generated noise from the brighter surround.

The intensifier tube is a quantum detector of high efficiency, and can provide images that appear "bright" even with extremely low levels of illumination; these images can be photographed with very short exposure times (0.1–0.01 second). However, a limitation results from the fact that the information content of the image is restricted by the number of quanta available to the cathode; images with photon-noise-limited information content have a grainy quality which makes them difficult to interpret, whether the image is photon-limited at some artificial photo-detector or at the retina itself (Rose, 1955, 1957).

Experience gained to date indicates that as applied to microscopy, the image intensifier is most useful in recording events producing or transmitting limited numbers of photons and with low ambient light, such as bioluminescence of single cells and subcellular particles, high extinction polarization and interference microscopy and fluorescence microscopy. It should also be useful for reducing the total light energy to which the specimen must be exposed, provided that photon noise does not adversely impair the quality of the image. The knowledge that images are limited by statistical fluctuations in photon flux permits the microscopist to choose and optimize the type of information—spatial, temporal or gray-scale—to be extracted by different recording procedures.

Some applications of the image intensifier to biological processes are described in an accompanying report.

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Application of an image intensifier tube to the microscopic observation of bioluminescent cells and visualization of weak radioactive source distributions. G. T. Reynolds, R. D. Allen, R. Eckert, J. W. Hastings and S. Inoué.

The image intensifier described in the accompanying paper has been applied to microscopic observations of bioluminescence in *Noctiluca miliaris.* Two series of observations were made. In the first, a preparation was placed on the stage of a Reichert phase contrast microscope, in a shallow channel with excitation electrodes spaced 1–2 cm. apart. Single and multiple electrical pulses of the order of 10 volts were used to stimulate bioluminescent flashes, and the resulting images were recorded photographically at the output of the image tube. Dark field pictures were taken for structural comparisons. The luminescence appears to come from the surface of the cell, and structural sources of the order of 1–2 μ were followed with an oil immersion objective (N.A. 1.25) in successive bioluminescent flashes induced at 20-second intervals.

In the second series of observations, an American Optical Co. rectified polarizing microscope was used at different magnifications. The intensifier system provided ample gain for photographing individual flashes of *Gonyaulax polyedra* stimulated by exposure to acetic acid. So far, the motion of the organisms has prevented close spatial identification of the light sources with structural features shown in photographs obtained through crossed polarizers.

The image intensifier was also applied to the photographic recording of weak radioactive sources. One-μ curie sources of P^{32} and of C^{14} occupying *ca.* 10 mm.^{2} were deposited on a cover glass. The sources were covered with a thin sheet of plastic scintillator 0.0004 inch thick. The β particles of the radioactive decay give rise to light in the scintillator; the latter is imaged on the photocathode by a pair of f/1.9 lenses. The output of the image tube was bright enough that 10^{-4} μ curie could have been photographed in one second.

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The effects of antibodies to single-stranded deoxyribonucleic acids on the embryonic development of Arbacia punctulata. HERBERT S. ROSENKRANZ.

The effects of rabbit antibodies with specificities for single-stranded deoxyribonucleic acids (DNA) on the embryonic development of *Arbacia* were investigated. Using fluorescein-tagged antibodies, it could be shown that these were able to penetrate into both the fertilized and unfertilized sea urchin egg. Similarly tagged non-specific gamma globulins did not localize in these eggs. The addition of immune sera to fertilized eggs resulted in either (a) an inhibition of the first cleavage, (b) abnormal early cleavage, (c) an arrest at the blastula stage, or (d) the development of abnormal plutei. These effects were found to depend on the immunochemical specificity of the antibodies and the dilutions used. Pre-immunization sera were found to be without any effect. Seven different antisera (each tested five times) with specificities for either the purine, pyrimidine, nucleoside or nucleotide moieties of DNA were thus studied.

The results suggest that single-stranded DNA is a physiologically important intermediary in the replication and differentiation of fertilized sea urchin eggs.

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The microscopy of ATP-reactivated ciliary models. PETER SATIR AND F. M. CHILD.

Models of ciliated cells of frog palate epithelium (*Rana pipiens* or *R. catesbiana*) have been prepared by appropriate extraction in glycerine solutions. The cilia of such models can be reactivated after washing by the addition of 5×10^{-3} M ATP. They generally beat synchronously on a given cell. Electron microscopy of such preparations shows ciliary membranes that are swollen or broken. Often no membrane is present at all. Unextracted controls normally have intact membranes and the usual $9 + 2$ axial filament complex, which is separated from the membrane by a structureless outer zone. Glycerination apparently entails (1) swelling of the outer zone, followed by (2) membrane fragmentation and disintegration, (3) freeing of the filament complex, (4) swelling of the complex, and finally (5) dislocation of the peripheral filaments. Most of the reactivatable cilia are probably in stages 2–4 where there is extraction of matrix and filament material, but the $9 + 2$ pattern is preserved. This suggests that (1) ciliary movement can occur in the absence of an osmotically-active membrane and (2) the active contractile elements of the ciliary shaft are localized within the axial complex. Models of reactivated mussel gill (*Modiolus*) whose lateral cilia beat with a slow metachronism are being studied to confirm these suggestions.

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Chemical and biological characteristics of growth-inhibiting agents from Mercenaria mercenaria extracts. SISTER M. ROSARII SCHMEER, O.P.

Growth-inhibiting agents in extracts of *Mercenaria mercenaria* indicate their presence by antitumor activity on the Sarcoma 180(Sa 180) and Krebs 2 ascites tumor, propagated in the form of ascites and implanted as solid tumor, in Swiss albino mice.

Out of a series of mollusks tested (paper in press), *Mercenaria* yields the most promising material. Properties of its active agent are described. In early experiments *Mercenaria* extracts inhibited the growth of Sa 180 in dilutions in which they had no toxic effect. While all untreated Swiss mice (controls) died within ten days, the treated animals (100%) survived 6 months, after which time they were sacrificed. They produced normal litters and evidenced no tumor recurrence.

In present investigations extracts of *Mercenaria* were found to be active also against the

Krebs 2 tumor in the laboratory of Dr. Albert Szent-Györgyi, Institute for Muscle Research, Marine Biological Laboratory, Woods Hole, Massachusetts.

Extracts in higher concentrations are toxic. Toxicity is perhaps due to potassium present which can be eliminated by dialysis. A "unit" of antitumor activity can be extracted from 80–100 mg., wet weight, of *Mercenaria*. Approximately 10,000 units can be obtained from 1 kg. wet weight of *Mercenaria*. The active agent or agents can be extracted with water. There is no precipitation of antitumor agent by 20% saturation with ammonium sulfate. Most of the activity precipitates between 25% and 70% saturation with ammonium sulfate. The active principle is thermostable, and lyophilization at −65° F. does not destroy antitumor properties. Partial purification on Sephadex G-100 columns suggests a molecular weight of less than 100,000 g.

It is hoped that with further studies the active agent or agents may be isolated, purified and lead to application in cancer as a therapeutic and prophylactic agent.

This work has been partially supported by National Science Foundation Fellowship 73182.

Pharmacological studies relating to chemical mediation at the chromato-neural junction in the sand flounder, Scophthalmus aquosus. GEORGE T. SCOTT AND JAMES C. HICKMAN.

Evidence based on neurological investigation indicates that the sand flounder, unlike other teleost fishes, possesses mononeuronic control of chromatophores (*i.e.*, one set of fibers causing aggregation of pigment). An extremely sensitive and highly reliable area for study of local action of drugs on skin chromatophores was found to be the opercular region. Subcutaneous injections of 0.1 ml. were made. All values are expressed as micrograms.

The ED (effective doses) for drugs producing pigment aggregation were as follows: epinephrine 0.04, norepinephrine 0.04, metamphetamine 0.04, serotonin 0.04, isoproterenol 0.08, dichloroisoproterenol 7, phenelzine 0.3, pheniprazine 0.5, etryptamine 2, iproniazid and isocarboxazid were inactive. The active drugs are all substituted aromatic ethylamines.

Drugs producing pigment dispersal fell into several classes. The ED were as follows: (tranquilizing) fluphenazine 0.08, thiopropazate 0.1, prochlorperazine 0.7, chlorpromazine 0.8, thoridazine 2, promazine 3, trifluoperazine 4, acetophenazine 5, mepazine 5, promethazine 6, triflupromazine 7, levomepromazine 8, chlorpromazine sulfoxide 30, chlordiazepoxide 70, meprobamate 80, reserpine (inactive at 20); (energizers) amitriptyline 1, imipramine 2, tranylcypromine 20; (serotonin blockers) L.S.D. 0.8, methysergide 2; (adrenergic blocking) Dibenamine 0.2, Dibenzyline 5; (sedatives) dibucaine 0.2, meperidine 10, pentobarbital sodium 80, secobarbital sodium 100, phenobarbital (inactive at 100).

Pretreatment with pyrogallol, an inhibitor of catechol O-methyl transferase (COMT), had a marked effect on the action of certain of the drugs. Five mg./kg. was injected interperitoneally prior to drug testing. The ED for epinephrine and norepinephrine fell from 0.04 to 0.0001 and 0.00008, respectively, while serotonin rose from 0.04 to 2. Iproniazid, a monamine oxidase inhibitor, on the other hand, had no effect on the ED for epinephrine. Pyrogallol had an effect on only three of the eleven drugs studied which produced dispersion. One was the adrenergic blocker Dibenamine (ED raised from 0.2 to 9). The others were the two most potent phenothiazine tranquilizers, fluphenazine and thiopropazate with ED values raised from 0.08 to 7 and 0.1 to 10, respectively.

The data strongly suggest the following: (1) A catechol amine is the chemical transmitter. (2) COMT is an enzyme involved in neuro-chromatophore physiology. (3) Two of the most potent dispersing agents are phenothiazines acting as adrenergic blocking agents. (4) The other pigment-dispersing drugs may act in a less specific manner on the chromatophores.

This investigation was supported by PHS Grant NY 3903 from the National Institute of Mental Health to Oberlin College.

Specific inhibitory control of regeneration in Clymenella torquata. STEPHEN D. SMITH.

Sea water extracts of the anal collar of the 22-segment maldanid worm, *Clymenella torquata*, when applied to freshly isolated individual posterior segments (17 to 19), inhibit the normally occurring regeneration of new tail tissue for at least ten days. Similar extracts of prostomia inhibit the regeneration of heads from isolated anterior (4 and 5, and 6 and 7) two-segment pieces. The inhibitors are strictly homologous in action—prostomial extracts do not affect tail regeneration, and anal collar extracts do not affect head regeneration. In addition, extracts of dorsal and ventral halves of the anal collar may inhibit specifically their respective portions of a regenerating tail. There is, however, evidence of considerable cross-reaction of the dorsal and ventral inhibitors.

The tail-inhibitor has been found to be distributed along a posterior-to-anterior gradient of activity.

Both head- and tail-inhibitors are electrophoretically mobile and positively charged at sea water pH (*ca.* 8.1).

Present evidence indicates that the tail-inhibitor, at least, blocks an initial step in regeneration, being effective for at least ten days after only three hours of contact with a freshly isolated posterior test segment.

Ultraviolet microbeam disruption of sarcomere structure. RAYMOND E. STEPHENS.

An effort was made to differentiate between the classical Huxley sliding filament model for muscle contraction and alternative models in which the actin filaments are thought to be connected to the myosin filaments at the M band of the sarcomere. Glycerinated resting length rabbit psoas myofibrils, isolated in low salt buffer, were irradiated with monochromatic ultraviolet light and subsequently contracted with ATP. The apparatus employed was a polarizing microscope equipped with a reflecting condenser and a Wild illuminator with HBO 200 mercury lamp and quartz optics. The UV microbeam, approximately 1 μ in width, was formed by focusing a slit image in the specimen plane and irradiating through the slit area with monochromatic light, obtained by the use of interference filters. The contraction, produced by allowing 0.1% ATP solution to diffuse into the preparation, was observed and photographed under a Zeiss phase contrast microscope. Irradiation in the I band apparently disrupts the actin filaments, for it prevents contraction on the irradiated side of the sarcomere; the optimum wave-length for this action was found to be 260 mμ. Ultraviolet light of 280 mμ wave-length was found optimum in causing loss of birefringence but not loss of material from the A band, thus implying a disordering of the myosin filaments. Sarcomeres in which two-thirds of the A band had been irradiated with 260 mμ UV showed contraction on the non-irradiated side, indicating that for contraction no attachments are necessary between the actin filaments (disrupted at this wave-length) in one half of the sarcomere and the myosin filaments in the M region.

Phenols in the cuticle of the fiddler crab, Uca pugnax, and their possible function in sclerotization. J. ROSS STEVENSON.

Several investigators have reported the presence of various phenolic substances in insect cuticles and presented evidence that these substances are oxidized to o-quinones, which strengthen and harden the cuticles by linking together protein chains. Although crustacean cuticles are usually hardened by calcification, the presence of oxidases in crustacean cuticles suggests that phenolic hardening (sclerotization) occurs also.

As a preliminary investigation of this possibility, an attempt was made to find phenols in cuticles of the fiddler crab and to find changes in concentration of such phenols with degree of hardness of the cuticles. The scraped and rinsed carapaces of specimens of *Uca pugnax* at intermolt stages A_1, A_2, and C_4 were extracted with ethanol and water, and the water extracts were extracted with ether before and after acidification. Thin layer chromatography on non-activated silica gel was used to separate the substances present in the extracts.

Six substances that would couple with diazo-p-nitroaniline were found in the concentration range of about 0.1 to 10 mg. per carapace. One of these was present only in stage A_1; three were present in stages A_1 and A_2 but absent at stage C_4; one was present at both A_1 and C_4 but about 5 times as concentrated in A_1 as in C_4; and one was in approximately equal concentration in stages A_1, A_2, and C_4. All of these substances, and particularly those that disappear or decrease in concentration, might be involved in sclerotization.

Each of these substances was characterized by its solubility, its R_f in n-butanol:acetic acid: water (4:1:5), and its reactions with various reagents.

Successive stages in the life-cycle of Homalometron pallidum, a digenetic trematode from the intestine of Fundulus heteroclitus. HORACE W. STUNKARD.

The name *Homalometron pallidum* was proposed by Stafford, 1904, for a digenetic trematode described but not named by Linton, 1901, from *Fundulus heteroclitus* taken in the Woods Hole area. *Homalometron* was included in the family Allocreadiidae until Cable and Hunninen, 1942, designated it as type of the subfamily Homalometrinae (emended, Homalometroninae by Manter, 1963) in the family Lepocreadiidae. Cable and Hunninen suppressed *Anallocreadium* Simer, 1929 as a synonym of *Homalometron*.

Cercariae liberated from *Hydrobia minuta* are very similar to others from *Amnicola peracuta* described by Sewell, 1937, as the larvae of *Anallocreadium armatum*. These cercariae are produced in rediae and encyst as metacercariae in *Gemma gemma* and small polychaetes. A series of specimens, from very small juveniles to mature, ovigerous *H. pallidum*, were taken from the intestine of *F. heteroclitus*. Eggs from these worms were incubated in sea water and later fed to laboratory-raised *H. minuta*. Six weeks later these snails were liberating cercariae, identical with those of natural infection.

Aided by a grant from the National Science Foundation, G-23561.

Events of the cardiac cycle in Mustelus canis. FREDERICK N. SUDAK.

Intra-atrial, intraventricular, ventral aortic, and intrapericardial pressures were measured simultaneously in order to investigate further the role of the pericardium in the cardiodynamics and energetics of the circulatory system in the dogfish *M. canis*. Animals were immobilized by means of a mid-diencephalic section and destruction of the spinal cord below the twentieth vertebra. Pressure measurements were obtained directly from the heart chambers, pericardium, and ventral aorta by means of 18-gauge needles or 0.044″ catheters attached to pressure transducers. Pressures below atmospheric pressure found in the pericardial cavity varied during the cyclical activity of the heart. Intrapericardial pressure averaged 2.4 mm. Hg (range, 1.0 mm. Hg to 4.7 mm. Hg) below atmospheric pressure just prior to atrial contraction and 5.6 mm. Hg (range, 1.8 mm. Hg to 10.0 mm. Hg) below atmospheric pressure just after the peak of ventricular systole. End-diastolic pressure of the atrium averaged 2.0 mm. Hg (range, 0 mm. Hg to 3.7 mm. Hg) below atmospheric pressure and 2.9 mm. Hg (range, 0.2 mm. Hg to 8.4 mm. Hg) below atmospheric pressure just after the peak of ventricular systole. End-diastolic pressure in the ventricle averaged 0.6 mm. Hg (range, 0 mm. Hg to 3.2 mm. Hg) below atmospheric pressure and 1.9 mm. Hg (range, 1.0 mm. Hg to 5.2 mm. Hg) during early diastole. After the atrio-ventricular valve closed following atrial ejection, the atrial pressure remained below ventricular pressure until the next atrial contraction occurred.

These data support the hypothesis that part of the energy of contraction in the dogfish heart is stored within the pericardial cavity as potential energy. This energy is imparted to the atrium and may be used to aspirate blood back to the heart during the diastolic phase of the cardiac cycle. Furthermore, these data suggest that the atrium alone is capable of aspiratory activity while the ventricle is dependent upon atrial contraction for its blood supply.

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Studies of melanin biosynthesis in the ink sac of the squid (Loligo pealii). III. *In vitro culture of tissues and isolated cells from the adult ink gland.* GEORGE SZABÓ AND JOHN M. ARNOLD.

This paper is an extension of earlier results, previously reported here by us (1961, 1962).

The techniques used here were based on tissue culture techniques known to be successful for the growth of embryonic organs, tissues, and reaggregated cells of this species. The ink glands of adult squid were dissected out from the sac, washed in several changes of sterile sea water to remove the excess ink. They were either dissociated in 0.5% trypsin in Gatenby's marine molluscan saline for 45 minutes at room temperature, or isolated "leaflets" of the organ were explanted. These cells or tissues were placed in one of the following media: Adult squid blood (45%), sterile sea water (45%), antibiotic-phenol red solution (10%); 1066 medium (Cappel Laboratories) diluted with sea water; 199 medium (Cappel Laboratories) diluted with sea water; sterile sea water (80%) horse serum (Cappel) (10%); antibiotic-phenol red solution (10%). The cultures were grown in either disposable plastic tissue culture flasks (Falcon), Cooper dishes (Falcon) or in flying cover glass preparations maintained at +18° C.

It was possible at least to maintain whole tissues from the ink gland in all of the culture media for periods up to 20 days. It is not known as yet whether mitosis was occurring in these tissues. However, in horse serum in the blood-sea water medium, fibroblast-like cells migrated from the tissue and became attached to the substrate. Many of the tissues showed considerable darkening that appeared to be melanization. Muscular contraction continued in most of these tissues at least 20 days.

The trypsinized cells survived only in the blood-sea water medium. The cells first appeared as fibroblasts and later formed large aggregates which seemed to melanize.

It is hoped that these culture methods will be used for the study of *in vitro* synthesis of melanin and other pigments occurring in the squid.

Studies of melanin biosynthesis in the ink sac of the squid (Loligo pealii). IV. Biochemical studies of the ink gland and the ink. GEORGE SZABÓ AND GEORGE WILGRAM.

The ink gland was homogenized and centrifuged, first at low speed, later at 12,000 *g*. The pellet was re-suspended in a sucrose gradient and centrifuged again. The ink from the ink gland was treated in the same way (without previous homogenization). As control, the eye of the squid was also homogenized and treated the same way.

The fractions were incubated in a Warburg apparatus and their tyrosinase and succinic oxidase activity were estimated. In the case of the ink gland the tyrosinase activity was high in the supernatant and high in the fraction #1 of the gradient. It was also present in fraction #2 and in the pellet. In the ink the tyrosinase activity was high in the supernatant, low but present in the sucrose gradients. In the eye there was some tyrosinase activity in the pellet. Succinoxidase activity was absent in the supernatants of the ink gland, or eye, it was present in fraction #3 of the ink gland, it was high in the #4 fraction of the gland (mitochondrial fraction), also high in the upper fraction of the eye gradient. Its amount was low in the supernatant of the pure ink.

These results suggest that there is a free tyrosinase present in the ink sac of the squid. Succinoxidase is absent in the ink granules, in contrast to mitochondria. The role of the tyrosinase in the eye is subject to further study, as the pigment of the eye is not melanin, but ommochrome.

Recovery of small axon activity following freezing of squid nerve. ROBERT E. TAYLOR.

Whole nerve trunks from the mantle of the squid, *Loligo pealei,* were cooled to temperatures of −30° C. either in a few cc. of solution, adhering to a stainless steel holder or in stoppered glass tubes, by immersion in the gas phase above liquid nitrogen or dry ice. Activity was tested by action potential recording, using an external resistance discontinuity. Rates of cooling were controlled by adjustment of the position of the holder in a covered Dewar flask; temperature was measured with a thermistor in contact with the nerve and continuously recorded. Rates of cooling from one-half degree per minute to about 10 degrees per minute were tried; thawing rates varied from about 2 to 20 degrees per minute. Glycerol, dimethyl sulfoxide and ethylene glycol, 5 and 10 per cent by volume, were tried as protective additives, with natural and artificial sea water.

None of the combinations tried resulted in recovery of electrical activity in the giant axon following cooling to −3° C. or lower. A group of fibers with conduction velocity about 5–6 mm./msec. recovered quickly after cooling to −17° with 10% glycerol added and in one-half to one hour after thawing with 5% or 10% dimethyl sulfoxide. A group of fibers with conduction velocity about 1.5 mm./msec. recovered from −30° C. without additive.

The axoplasm of frozen and thawed axons appeared normal to sight and touch.

The relationship between light and photosynthesis in intertidal benthic diatoms. W. ROWLAND TAYLOR AND JOHN D. PALMER.

Many intertidal benthic diatoms migrate to the surface at low tide. The relationship between light and photosynthesis is of ecological interest and studies were made of diatom populations on the sand flats of the Barnstable Harbor. Squares of nylon bolting, 200 micron mesh, were spread on the diatom patches. Diatoms migrate through the cloth and when rinsed into filtered sea water, suspensions of about 50,000 cells per milliliter were obtained. Species composition depended upon the location, but for any one collection, was almost unialgal. The suspension was dispensed into a series of 30-ml. bottles and to each was added C^{14}-labeled sodium carbonate. Using plastic screen light attenuators, the diatoms were incubated under light conditions varying from 1% to full mid-day sunlight. The temperature was the same as that of the exposed sand. Solar radiation was measured with a pyroheliometer. After incubation, biological activity was stopped by the addition of iodine-potassium iodide. The diatoms were collected on Millipore filters and assayed for C^{14} uptake. The results were corrected for dark uptake, which amounted to only 1% of maximum carbon fixation. The diatoms were very efficient at low light levels, 35% of maximum photosynthesis being reached at 0.75 langley per hour or about 1% of mid-day solar radiation. Maximum photosynthetic rate was obtained at about 12 langleys per hour. There was only a 10% inhibition as the light level increased to 75 langleys per hour. Measurement of light penetration through the sand in which these diatoms are found showed 1% reached a depth of 3 millimeters. Diatoms 3.5 mm. deep are above their compensation level and diatoms from 1.5 mm. up photosynthesize above 90% of their maximum capacity.

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Rapid exchange of D_2O and H_2O in sea urchin eggs. ROBERT W. TUCKER AND SHINYA INOUÉ.

When *Arbacia punctulata* and *Lytechinus variegatus* eggs from normal (H_2O) sea water are layered on top of a heavy (D_2O) sea water solution (concentration of D_2O greater than 98%) and then centrifuged at 200 *g* for two seconds, the eggs fall even though the density of the D_2O sea water (1.127 gm./ml.) is greater than that of the eggs in H_2O sea water (1.084 gm./ml. for *Arbacia punctulata*). The converse experiment of eggs which have been equilibrated in D_2O sea water falling under gravity in H_2O sea water (1.02 gm./ml.) shows that the eggs fall at a large initial rate, gradually slowing down to the terminal velocity determined by Stokes' law. Eggs in H_2O sea water when forced under D_2O sea water rise for a maximum of 3.5 seconds and then fall under gravity. All three experiments can be explained by the fact that at least 30% of the heavy D_2O exchanges reversibly for the light H_2O in less than two to three seconds.

Multiple molecular forms of malate dehydrogenase in separated blastomeres of Arbacia embryos. CLAUDE A. VILLEE AND RICHARD O. MOORE.

There are five electrophoretically separable L-malate dehydrogenases (MDH) in unfertilized *Arbacia* eggs but only three in 6-hour embryos. This phenomenon appeared to offer an opportunity to study the differentiation of enzymes in the cells of developing embryos. Eggs collected by electric shock were fertilized and grown four hours at 23° (64-cell stage). The embryos were frozen briefly, then treated three minutes at 37° with 0.53 *M* NaCl containing 2×10^{-3} *M* ethylene diamine tetracetate, pH 5.0. Two volumes of 0.265 *M* Tris buffer, pH 7.9, containing 0.01% trypsin, were added and the mixture placed in an all-glass homogenizer at 37°. Slow excursions of the pestle were continued until microscopic examination revealed that all of the embryos were dissociated. The dissociated blastomeres were layered on a 0.29–0.87 *M* sucrose gradient and centrifuged at 750 rpm for 25 minutes. The large blastomeres formed a pellet at the bottom of the tube and the small blastomeres formed an interface between the 0.29 and 0.87 *M* sucrose. The layers were separated, the blastomeres were disintegrated by ultrasonication and the resulting preparations were assayed spectrophotometrically for MDH activity with diphosphopyridine nucleotide (DPN) and its analogues. Other aliquots were subjected to microelectrophoresis on polyacrylamide gel (70 minutes, 5 mA., Tris-glycine, pH 8.6). The resulting gels were stained for MDH activity using L-malate, DPN or analogue, phenazine methosulfate, nitro blue tetrazolium, KCN and glycylglycine buffer, pH 7.4. The MDH activity with acetyl pyridine DPN exceeded that with DPN by a ratio of 1.4 in small and 2.3 in large blastomeres. Staining the acrylamide gels revealed a slowly migrating band of DPN-MDH activity in small blastomeres which is not present in the large cells. The small cells had a total of three DPN-MDH's and the large cells had two. Two of the bands in the small cells appeared to be identical to the two bands in the large cells.

Relationships between nucleolar and ribosomal ribonucleic acids. W. S. VINCENT, ROBERT MUNDELL AND CONSTANCE HAMMOND.

Fractionation of nucleoli isolated from starfish oocytes has allowed for the demonstration of three categories of ribonucleic acids which differ with respect to metabolic activity, base ratios and molecular size. One of these categories, the *residue* fraction, contains an RNA which is identical in base composition to cytoplasmic ribosomal RNA.

When one examines electron micrographs of *in situ* and isolated nucleoli, one finds that the nucleoli contain large numbers of small granules which appear to be similar in size and morphology to ribosomes. We have not yet been able to isolate these granules, but the isolated nucleoli are able to support incorporation of phenylalanine in the absence of cytoplasmic ribosomes, thus suggesting a ribosome-like function.

In an attempt to determine whether or not the nucleolus may be the site of synthesis of cytoplasmic ribosomes, we have examined the RNA of nucleoli by sucrose gradient centrifugation and by serological means. The *residue* RNA is found to contain two major molecular populations of approximately 18S and 28S, identical with ribosomal RNA.

An antibody developed against whole nucleoli is shown to cross-react with both RNA and protein antigens. When RNA fractions of the oocyte are tested against the antibody, both nucleolar and ribosomal RNA show a high titer of reactivity, thus indicating considerable molecular similarity.

The above results strongly suggest nucleolar origin of ribosomal RNA, and lead to the prediction that the ribosomal RNA cistrons may be found in the nucleolus-associated chromatin.

Amino acids in the economy of the sipunculid worm, Golfingia gouldii. RAGHUNATH A. VIRKAR.

The general pattern of uptake of C^{14}-labeled glycine by *Golfingia* from sea water was studied. Uptake is linear with time for at least one hour, and is an exponential function of body weight, the exponent having a value of approximately 0.5. The rate of uptake is a function of ambient concentration; a double reciprocal plot of uptake against concentration gives a straight line, permitting the evaluation of a V_{max}, which is approximately 4.10^{-7} moles/hr./4-gram worm, and of a K_m, which is approximately 10^{-4} *M*/1. Uptake is not inhibited by KCN or 2,4 dinitrophenol at concentrations of 10^{-3} *M*, or in nitrogen-saturated water.

Sacs made from the body wall of *Golfingia* are capable of transporting glycine inwards against a concentration gradient. After four hours, the radioactivity inside the sacs was 5–6 times that outside. During the same period, the body wall accumulated radioactivity 30–40 times that in the external medium.

The distribution of free amino acids in *Golfingia* was investigated. Samples of body wall and coelomic fluid were extracted with perchloric acid and the extracts neutralized with KOH. The ninhydrin-positive material in the extracts was estimated colorimetrically and expressed as amino acids. In normal worms, the concentration in the body wall is about 0.5–0.6 mole/liter of tissue water, that in the coelomocytes 0.1 mole/liter of cells, and that in the fluid proper about 5–6 millimoles/liter of fluid. When the animals are subjected to a modest osmotic stress (80% sea water), the body wall concentration drops to about 0.3 M/l. More drastic changes in salinity (50% sea water) do not produce a further drop in this concentration. This response occurs more rapidly than the reported volume and weight regulation of *Golfingia* at moderately low salinities.

The effect on cleavage of Arbacia eggs of ultrasound applied to a small area of the cell surface. WALTER L. WILSON AND RONALD M. SCHNITZLER.

Fertilized eggs of *Arbacia punctulata* were subjected to ultrasound by means of a stainless steel needle which touched the cell surface. The needle was machined into the tip of a stainless steel cone which was glued at its base to one end of an electroded and polarized barium titanate cylinder. This transducer was activated at approximately 85,000 cycles per second. To hold an egg in a fixed position during sonation, a drop of eggs was distributed over a layer of 1% agar in sea water on a slide. The slide with eggs was mounted on the microscope stage and the needle tip was placed against a small area of the egg surface by means of a micromanipulator. Experiments were carried out with eggs in a moist chamber.

Eggs were subjected to sonic energy at various times after fertilization, and treatment was maintained for various durations. The amplitude of vibration was adjusted until there was a gentle churning of the cytoplasm. If sonation is initiated at some time during the first mitotic cycle, cleavage does not occur as long as treatment is continued. However, if the sonic amplitude is low and the churning of the cytoplasm very gentle, there is an abortive cleavage. When treatment is stopped prior to cleavage time of control eggs, the treated egg cleaves, but the cleavage is delayed. If treatment is continued beyond the cleavage time of normal eggs, but not prolonged, cleavage occurs after a short interval following cessation of sonation, but typically cleavage is abnormal. When, during the second mitotic cycle, churning is produced briefly in one of the two daughter cells, cleavage of the treated cell occurs later than that of the other cell. Typically cleavage of the treated cell is abnormal.

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Occurrence, distribution, and storage function of lysosomes in renal tissue of a teleostean fish, Centropristes striatus. PHILIP ZEIDENBERG AND AARON JANOFF.

Lysosomes are present in renal tubular epithelial cells of mammals where they are known as renal droplets and appear to serve a detoxicating or protein storage function (Straus). Renal tissues of four marine species: a glomerular teleost (sea bass), an aglomerular teleost (toadfish), an elasmobranch (dogfish), and an invertebrate (lobster green gland) were assayed biochemically for three of the granule-bound acid-hydrolases known to be contained in mammalian kidney lysosomes. In the sea bass, toadfish, and lobster, differential centrifugation of tissue homogenates, followed by enzyme assays performed on nuclear, mitochondrial (lysosomal), microsomal, and cell-sap fractions of the homogenate, revealed the presence of acid-phenolphthalein-phosphatase, acid-RNAase, and beta-glucuronidase with highest specific activity in the mitochondrial (lysosomal) fraction. Dogfish kidney (and liver) possessed highest activity for all three enzymes in the cell-sap, a result which was not surprising in view of the relatively high concentration of vitamin A in body fluids of this and related species. In view of the atypical structure of lobster green gland and toadfish kidney (open ciliated tubules in the former, lymphorenal tissue in the latter) only sea bass kidney was chosen for additional experimentation. Histochemical staining for acid-phenyl-phosphatase was carried out by Barka's method and revealed a tubular epithelial localization of enzyme in large granules quite similar to the renal droplets described in mammalian kidney tubules. Furthermore, intravascular injection of a foreign protein (horse radish peroxidase) into these fish was followed by sequestration of the protein in the renal droplets of tubular cells (benzidine stain) in the manner described by Straus for rat kidney. Thus, sea bass kidney closely resembles rat kidney with regard to occurrence, distribution, and protein storage function of lysosomal granules.

The effects of hydrostatic pressure on the cleavage schedule of Arbacia eggs. ARTHUR M. ZIMMERMAN, LESTER SILBERMAN AND SONDRA C. CORFF.

Fertilized eggs of *Arbacia punctulata* were subjected to hydrostatic pressures of 5000–10,000 lbs./in.^{2} at 20° C. for durations of 1–30 minutes. The eggs were permitted to recover at atmospheric pressure and their division cycle and subsequent development were studied.

Pressure pulse treatment of 7500 lbs./in.^{2} for durations of 1–5 minutes produced delay in first cleavage which exceeded the duration of the pressure pulse. Subsequent development, however, was essentially comparable to the controls after 2–3 days. The extent of cleavage delay was dependent upon the stage of the mitotic cycle at which pressure was applied. Pressure of 7500 lbs./in.^{2} for one minute at prophase (39–43 minutes after insemination) resulted in a maximum cleavage delay of 12.7 ± 0.9 minutes (control cleavage schedule adjusted to 60 minutes). At syngamy (9–13 minutes) and at early streak stage (24–28 minutes) the delays were at their lowest, 2.7 ± 1.2 and 2.9 ± 0.9 minutes, respectively. Following syngamy (14–18 minutes) and at metaphase (49–53 minutes), intermediate delays of 7.4 ± 1.4 and 7.8 ± 1.2 minutes, respectively, were encountered. Unfertilized eggs, similarly pressurized, showed no consequential delays after insemination.

Pressure treatments of longer duration have a more marked effect on development. Less than 3% of the eggs developed to plutei following a 30-minute pressure treatment at 10,000 lbs./in.^{2}, while pressures of 7500 and 5000 lbs./in.^{2} produced less drastic effects on development.

Isolation of the mitotic apparatus from pressure-treated metaphase and anaphase eggs confirms earlier reports that there is a marked disorganization of the spindle and the aster following high hydrostatic pressure. The results are discussed in relation to the solating action of pressure on protoplasmic gel structures and their role in division.

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OBSERVATIONS ON DAILY AND TIDAL RHYTHMS IN SOME FIDDLER CRABS FROM EQUATORIAL BRAZIL[1]

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Experimental studies on daily and tidal rhythmicity in the fiddler crab, *Uca,* have been confined for the most part to North American populations of *U. pugnax* and *U. pugilator.* Both species possess a conspicuous daily rhythm of melanophore activity which consists of diurnal darkening and nocturnal lightening of the integument. This rhythm persists for weeks in the laboratory in darkness, and its frequency is independent of temperature level over a range of at least 20° C. (Brown and Webb, 1948). Superimposed upon the daily rhythm is a persistent tidal rhythm with a mean 12.4-hour period which gives rise to two suppplementary dispersions of melanin during each lunar day of 24.8 hours (Brown *et al.,* 1953). Periodic reinforcement of the maxima of the daily and tidal components when they come into synchrony every 14.8 days produces a semimonthly rhythm of darkening.

The phases of the tidal rhythm are determined by the time at which the crabs emerge from their burrows after being uncovered by the receding tide (Fingerman *et al.,* 1958). Crabs inhabiting burrows near the high tide level emerge earlier than, and show tidal darkening before, crabs living near the low tide level. Accordingly, phase differences have been reported among species (*U. pugilator, U. speciosa* and *U. minax*) inhabiting different levels of the beach (Fingerman, 1956; Fingerman *et al.,* 1958).

In metabolic rate *U. pugnax* and *U. pugilator* each display superimposed diurnal and tidal rhythms which persist in unvarying conditions in the laboratory (Brown *et al.,* 1954). These metabolic rhythms reflect diurnal and tidal rhythms of spontaneous motor activity (Bennett *et al.,* 1957). Generally the maximum of diurnal activity occurs during the early morning hours, and the maximum of tidal activity, near the time of low tide. A review of the studies which describe

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persistent rhythms in *Uca* and which analyze the mechanisms of timing and phase adjustment has been provided by Brown (1962).

Except for the reports of tidal phase differences in melanophore activity, little has been accomplished in the way of a comparative study of persistent rhythmicity at the species level. On the other hand, a framework for such an analysis has been provided by Crane (1941, 1943a, 1943b, 1944, 1957, 1958) in her field studies of behavior in more than fifty species of *Uca* distributed throughout the world. She has reported species differences in the phases of daily and semimonthly rhythms in social behavior. These phase differences are not correlated with habitat as are the species differences in the tidal melanophore rhythm, but rather they appear to be related to phylogenetic position. Furthermore, her descriptions of the diversity of habitats occupied by *Uca* species, ranging from far up tropical rivers to exposed ocean tidal flats, suggest that tidal rhythms must play roles of varying importance in the biology of fiddler crabs. Thus it appears that an extension of the studies on persistent rhythmicity to other members of the genus will yield additional information on the properties of daily and tidal rhythms. At the same time such studies should provide information on the extent to which persistent, or clock-timed, rhythms participate in the regulation of complex behavior patterns.

The following observations were made on some local species of *Uca* in November and December, 1962, during a stay at the Museu Paraense "Emilio Goeldi" in Belém, Brazil. Color change rhythms were observed in *U. mordax, U. rapax, U. maracoani,* and *U. thayeri.* Recordings of locomotor activity were obtained for *U. maracoani* and *U. mordax.*

COLLECTING SITES

Fiddler crabs were collected in the vicinity of Belém and Salinópolis in the state of Pará in northern Brazil.

Belém is situated on a point of land formed by the entrance of the Rio Guamá into the Rio do Pará, the estuary mouth of the Tocantins, at a distance of about 85 miles from the Atlantic Ocean. The geographical coordinates of the city are latitude 1°27′ S, longitude 48°30′ W. There is a large semidiurnal tide at Belém, the average range of the spring tides being 10.0 feet. Collections of *U. mordax* were made on the southern edge of the city from mud and concrete drainage ditches which empty into the Rio Guamá and which are subject to tidal flooding. Other collections of *U. mordax* were made from the muddy banks of a small tidal stream located a few miles away on the grounds of the Instituto Agronômico do Norte.

Salinópolis is situated on the northern seacoast about 90 miles northeast of Belém. It lies 45 miles from the equator at 0°39′ S latitude, 47°23′ W longitude. To the north of the town is an extensive tidal flat which is separated from the open ocean by offshore shoals. Here the range of spring tides is 15.9 feet. On Nov. 4 *U. maracoani* were collected for activity experiments from the barren mud flat near the middle of the tidal range. On Dec. 9 *U. maracoani* and the following crabs were collected for color change experiments: *U. rapax,* which were picked up near the high tide level where they had burrows in sandy soil among blades of grass; and *U. thayeri,* which were collected from burrows in a rocky clay bank amid tussocks of grass just below the mean high tide level. The habitats resemble ones typically reported for these species.

Rhythms in Color Change

Methods

In the laboratory, crabs were kept in a small amount of water in pans of white enamel or tinted plastic. For exposure to natural changes in illumination, pans were placed outside under the eave of the laboratory building where they were sheltered from direct sunlight and rainfall. Crabs to be maintained in constant light were placed in a windowless room illuminated by incandescent bulbs on a voltage-stabilized power supply. Within the room at the level of the pans light intensity was estimated at a few foot-candles. Air temperature in the room fluctuated around 29° C. with a mean daily range of 3° C.

Stage of melanophore activity was determined with a dissecting microscope according to the Hogben and Slome (1931) scale for describing the degree of dispersion. On this scale stage 5 indicates complete dispersion and stage 1, complete concentration. Melanophores on the anterior aspect of the merus of the first or second walking leg were observed. In *U. thayeri*, unlike the other species, the melanophores generally remained discrete and were seldom seen to coalesce. In addition, the stages of individual melanophores usually varied widely over the surface of the same leg. For this species only the central area of the merus of the second leg was staged. In the figures to follow each value represents the arithmetic mean of the sample. The size of samples varied because of availability of crabs. Both males and females were used.

U. mordax were collected from the Instituto Agronômico on Nov. 20 and divided into two lots, one being exposed to natural changes in illumination and the other to constant illumination. Melanophores were staged through the day in both lots on Nov. 26.

On Dec. 9 crabs collected at Salinópolis were sorted into pans and placed outdoors in natural light with the *U. mordax* remaining from the collection of Nov. 20. Starting at 4 AM on Dec. 12 all groups were staged at two-hour intervals until 8 PM on Dec. 13. On the morning of Dec. 14 the pans were placed in the constant-light room, and at 6 PM on Dec. 15 staging was begun and continued at six-hour intervals until 12 PM on Dec. 19. By Dec. 15 the original collection of *U. mordax* had so dwindled that it was replaced by a new lot of *U. mordax* collected at 5 PM on that day from the drainage ditches along the Rio Guamá. Standard time for Belém is used; the time meridian is 45° W.

Results

In Figure 1 are compared the average hourly stages of melanophores in two lots of *U. mordax* collected six days earlier, on Nov. 20, at the Instituto Agronômico. In the lot which had been exposed throughout to natural illumination, the rhythm of pigment migration passes through nearly the full range of concentration and dispersion. Periods of transition are sharp and occur at the times of sunrise and sunset. In the second group of crabs, which had been kept in constant low illumination, the rhythm of color change has been modified. The range of dispersion from mean minimum to mean maximum is much reduced; the periods of transition are very gradual; and the maximum dispersion no longer occurs during the daylight hours.

In Figure 2 are presented the daily rhythms in natural illlumination for three species from Salinópolis and the *U. mordax* still remaining from the collection of Nov. 20. Both *U. rapax* and *U. maracoani* possess overt rhythms whose general features are similar to those of *U. mordax*. By contrast the rhythm in *U. thayeri* is of very low amplitude.

During the staging it was obvious that the red chromatophores of *U. rapax* and *U. maracoani* were going through a daily variation roughly parallel to that of the melanophores. In *U. rapax* the red chromatophores were very evident, being numerous and resembling the melanophores in their dimensions.

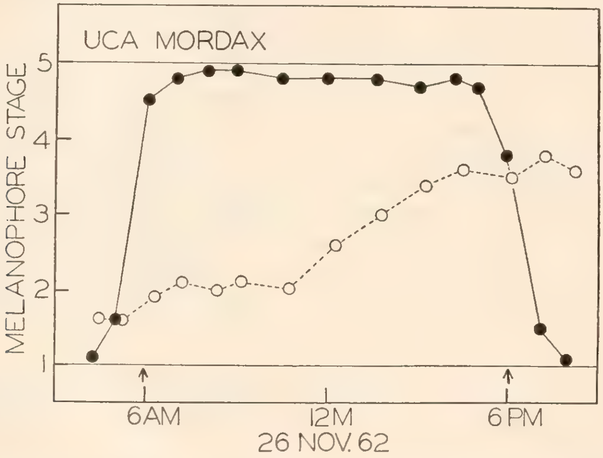

Figure 1. Melanophore rhythms in *U. mordax* after six days in experimental conditions. Closed circles are the average of 44 animals in natural illumination. Open circles are the average of 50 animals in constant low illumination. Arrows indicate times of sunrise and sunset at Belém.

The behavior of melanophores following transfer to constant low illumination is shown in Figure 3. The rhythm of color change persists for at least four days in *U. mordax* and six days in *U. rapax* and *U. maracoani*. In *U. thayeri* the amplitude is so much reduced that a rhythm is scarcely discernible.

The overt rhythms of *U. mordax*, *U. rapax*, and *U. maracoani* are common in several ways. In all cases the range of daily pigment migration is reduced below its range in natural illumination. For all three species the maximum and minimum levels of dispersion reached on each day remain fairly constant throughout the period of observation. Finally, inspection of the figure discloses that rhythms for two species have drifted out of phase with the outside daylight-darkness cycle. For *U. rapax* the maximum has shifted from noon to 6 PM in about six days. In *U. mordax*, where a similar phase shift has occurred, the results are essentially a repetition of those obtained in the earlier experiment (see Fig. 1), except for a change in amplitude.

Differences among the species occur in the daily levels of pigment dispersion and concentration. In *U. mordax* the melanophores fail to concentrate fully during the first night in constant light and for the following nights, but more apparent is their failure to disperse during the day phase. For *U. rapax* the melanophores fail to concentrate fully but do achieve nearly the maximum of dispersion observed in natural illumination. In *U. maracoani* there is failure both to concentrate and to disperse fully.

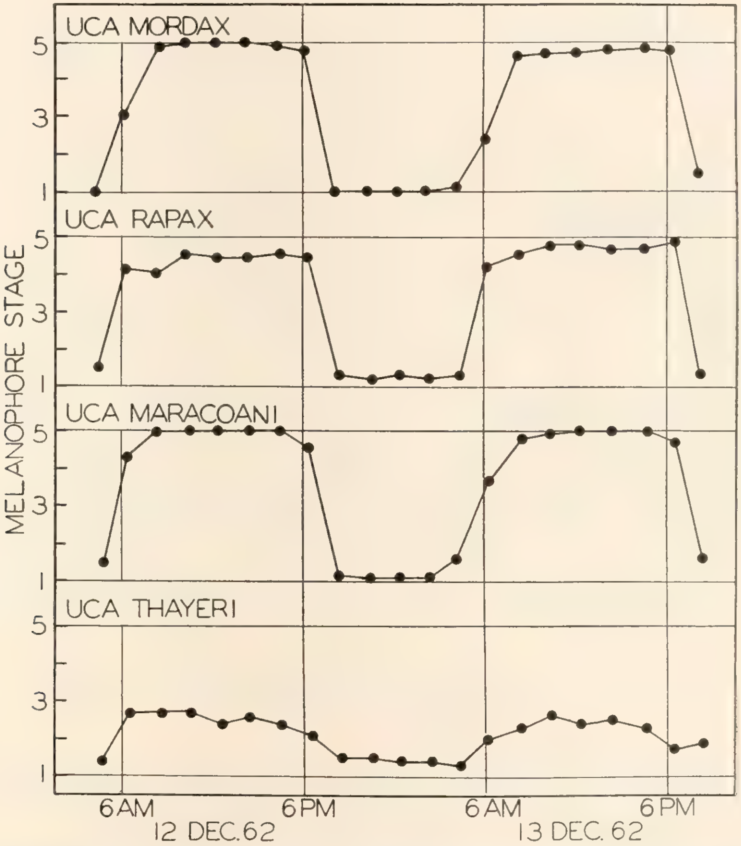

FIGURE 2. Melanophore rhythms in natural illumination. Number of crabs used were the following: *U. mordax*, 22; *U. rapax*, 30; *U. maracoani*, 26; and *U. thayeri*, 29.

Discussion

The overt rhythms of pigment migration in *U. mordax, U. rapax*, and *U. maracoani* exposed to natural changes of illumination are closely adjusted to the equatorial cycle of daylight and darkness, suggesting that the rhythms serve a camouflaging and protective function. *U. thayeri* does not exhibit an overt rhythm of color change, but it is notable that this species is characterized by patches of pile on the dorsal surface of the carapace and appendages. Possibly this morphological covering substitutes functionally for the melanophore rhythm.

The red chromatophores of *U. rapax* and *U. maracoani* undergo a daily rhythm of expansion and contraction when the crabs are exposed to natural cycles of illumination or to continuous low lighting. A daily rhythm has also been reported for the red chromatophores of *U. pugilator* (Brown, 1950). However, the rhythm in *U. pugilator* is evident only in uninterrupted darkness, since under conditions of illumination the stage of the red chromatophores depends primarily upon background. It is interesting that this rhythm is not salient in *U. pugnax* because until recently *U. rapax* and *U. pugnax* had been regarded as subspecies (Tashian and Vernberg, 1958). The rhythm in red chromatophore activity is thus a physiological distinction between the two groups.

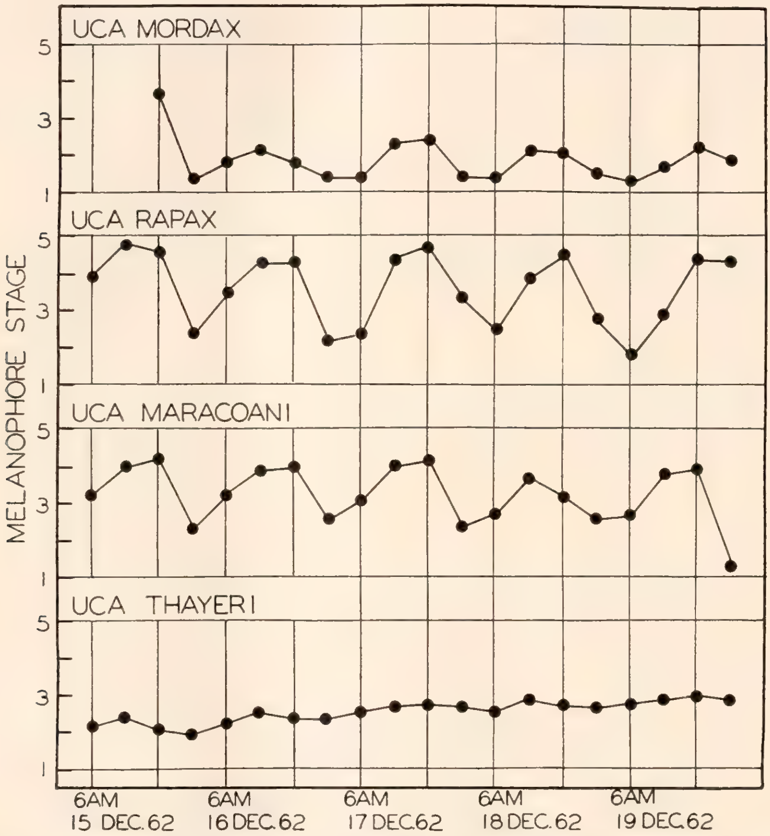

FIGURE 3. Melanophore rhythms in constant low illumination. Number of crabs used decreased during the experiment from 31 to 9 in *U. mordax,* 27 to 12 in *U. rapax,* 25 to 7 in *U. maracoani,* and 25 to 12 in *U. thayeri.*

Constant illumination clearly modifies the expression of the melanophore rhythm. Although the data of this study are very limited it is possible to make some comparisons with the results of a detailed series of experiments on the behavior of

U. pugnax melanophores in constant illumination (Brown and Hines, 1952). In *U. pugnax* constant illumination reduces the amplitude of the rhythm by inhibiting concentration during the night phase. For crabs on a white background the inhibition occurs progressively over several days. On a black background the maximum inhibition is achieved on the first day of the experiment.

The rhythm of *U. mordax* in constant light fails to resemble any pattern obtained for *U. pugnax* by Brown and Hines (1952). On the other hand, the suppression of the phase of diurnal dispersion in *U. mordax* is similar to the response of *U. pugnax* when it is transferred from a daily schedule of alternating light and darkness to uninterrupted darkness (Brown and Stephens, 1951). In *U. pugnax,* however, the initially inhibited diurnal dispersion regained its amplitude progressively over a period of days. This same phenomenon was observed also in a preliminary experiment with *U. mordax,* in which recovery of the amplitude of the rhythm was recorded over three consecutive days after the crabs had been placed in darkness. In *U. maracoani* the form of the rhythm resembles the one eventually attained by *U. pugnax* after several days at low light intensity on a white background. Whereas both *U. mordax* and *U. maracoani* were kept in white pans, *U. rapax* were kept in a green-tinted container. The response of these latter crabs resembles that obtained with *U. pugnax* on a black background at a light intensity of two-foot candles. In summary, constant illumination reduces the amplitude of the melanophore rhythm in fiddler crabs so far examined; but species differences occur in the manner by which reduction is effected.

In constant illumination the period of the melanophore rhythm in *U. mordax* and *U. rapax* exceeds 24 hours. A similar increase in period has been reported for *U. pugnax* in constant low illumination (Webb *et al.,* 1954). Deviation from a 24-hour period is commonly observed in many physiological rhythms when they are studied under constant illumination and temperature. The occurrence of these unnatural periods has been interpreted as an artifact of laboratory conditions produced by operation of the adaptive mechanism which ordinarily serves to adjust phases of the rhythm to natural light or temperature cycles (Brown, 1962).

Rhythms in Motor Activity

Methods

Spontaneous motor activity was recorded with an actograph consisting of tipping pans and a mechanical recording system somewhat similar to the one described by Bennett *et al.* (1957). On the evening of the day of collection crabs were placed individually with a few milliliters of water from the collecting site in aluminum pans with transparent plastic covers. Dimensions of the pans were 12 cm. in length, 6 cm. in width, and 4 cm. in height. The actograph was set within a small inner room of the laboratory where the recorded temperature averaged 30° C. and did not vary through more than 1° C. during any 24-hour period. The intensity of illumination was less than one foot-candle and was constant since the power was supplied from a voltage-regulated source. Activity of ten individuals was recorded simultaneously on a kymograph drum moving at the rate of 10 mm. per hour. Activity was measured from the records as millimeters of oscillation of the tipping pan per hour, and for each hour the average activity of all animals

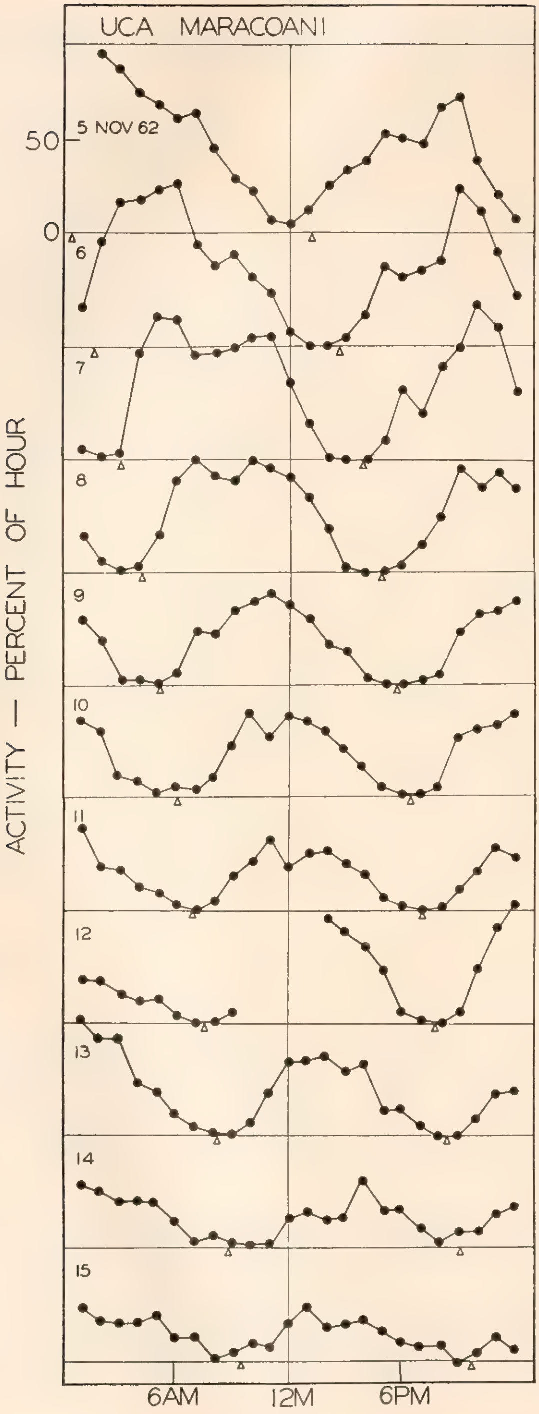
UCA MARACOANI
ACTIVITY — PERCENT OF HOUR
50
0
5 NOV 62
6
7
8
9
10
11
12
13
14
15
6AM
12M
6PM

FIGURE 4.

was computed. The activity was expressed as a percentage referring to the fraction of time in each hour that crabs were active. Each hourly value represents the amount of activity recorded during the preceding hourly interval; for example, the value for 6 AM is the measure of activity between 5 and 6 AM.

U. maracoani were collected at Salinópolis on Nov. 4 and recorded from Nov. 5 through Nov. 15. *U. mordax* were collected from drainage ditches along the Rio Guamá on Nov. 23 and recorded between Nov. 24 and Dec. 7. In both cases only large adult males were used. On Nov. 12, at the time of a power failure, the sea water in the pans of *U. maracoani* was changed. Except for this instance the crabs remained sealed in their containers throughout the period of recording.

Results

In Figure 4 are presented the mean hourly values recorded for *U. maracoani.* In its spontaneous activity *U. maracoani* possesses an overt tidal rhythm. On each day during the 11 days of study there occur two maxima and two minima which are synchronized with tidal events at Salinópolis. In the figure are indicated the times of high tide to which the minima of the activity cycle are closely tied. Although the amplitude of the activity cycles gradually decreases during the recording period it should be noted that this is due primarily to reduction in the height of the tidal maxima. The periods of almost complete inactivity at the time of high tide do not tend to disappear but are still present during the final days of recording. If the phases of individual animals had tended to drift apart the well defined periods of inactivity would have become obscured. Thus, there is no apparent loss of phase synchrony during 11 days among the ten animals under study. On Nov. 12 when the sea water in the aluminum pans was changed, there was a recovery for two or three cycles from the trend of decreasing amplitude.

It is often stated that the rate of tidal progression is 50 minutes per day, meaning that this is the interval between the recurrence of tidal events on successive days. Generally it is realized that this represents a mean value from which the actual interval may depart widely. As an example, on the first three days of the activity study, Nov. 5, 6, and 7, the average rate of progression of high tides at Salinópolis was 80 minutes per day. For the last five days, Nov. 11 through 15, the average rate had decreased to 41 minutes per day. Study of Figure 4 discloses that the crab activity cycles were also progressing across the day more rapidly at the beginning of the study than at the end, suggesting that the persistent activity of *U. maracoani* was following the actual rate of tidal progression rather than an average rate of 50 minutes per day.

In Figure 5A are presented the mean daily activity patterns for *U. mordax.* The recorded activity of these crabs is more variable than in the preceding case, and so, to facilitate analysis, the mean curves for each day have been smoothed with three-hour overlapping, or moving, averages. From inspection of the figure it is evident that the daily patterns for *U. mordax* are more complex than those of

FIGURE 4. Average daily patterns of spontaneous motor activity in *U. maracoani,* from November 5 through November 15, 1962. Values are expressed as the percentage of the hour that crabs were active. Triangles indicate times of high tide at Salinópolis where the crabs were collected. All tidal times were obtained from Tide Tables (1962) of the Coast and Geodetic Survey.

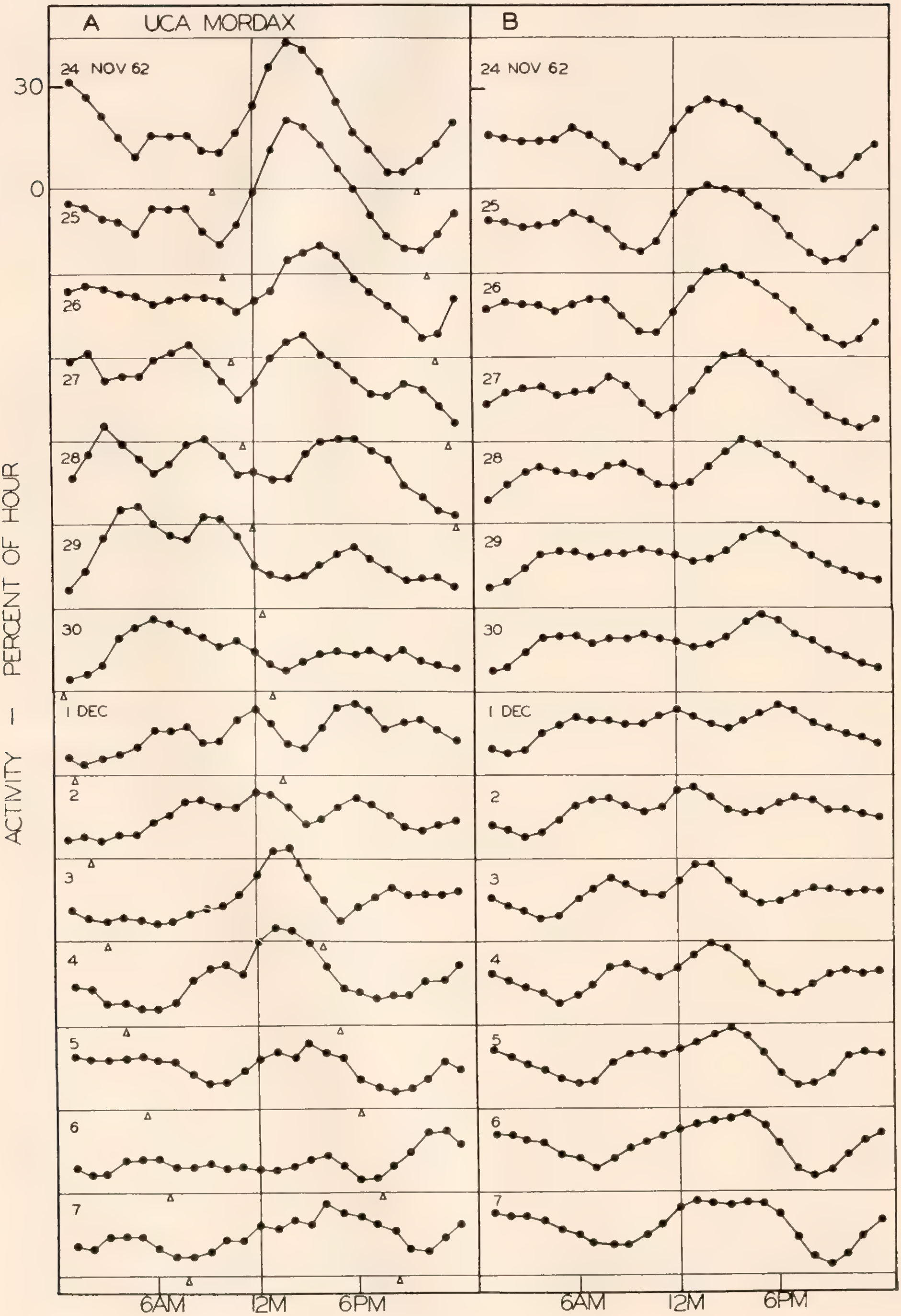
A
UCA MORDAX
B
24 NOV 62
25
26
27
28
29
30
1 DEC
2
3
4
5
6
7
30
0
ACTIVITY – PERCENT OF HOUR
6AM
12M
6PM

FIGURE 5.

U. maracoani. On the first two days of recording a large afternoon maximum occurs at the time of low tide, and two minima occur near the times of high tide. Thereafter the progression of a shifting, or phase-labile, component may be traced through successive daily patterns. For instance, one can follow the minimum which occurs at 9 to 10 AM on Nov. 24 and 25. On Nov. 27 the minimum is centered at 11 AM; on Nov. 29, at 2 PM; on Dec. 1, at 3 PM; on Dec. 3, at 5 PM; on Dec. 5, at 8 PM; and on Dec. 7, at 10 PM.

During the first five days of recording the average rate of progression for high tides at Belém was 36 minutes per day. Between Dec. 3 and 7 the average rate had increased to 67 minutes per day. It is clear from examination of Figure 5A that the phase-labile component of the activity rhythm is progressing slowly during the first days of the study and more rapidly during the period when the rate of actual tidal progression is increased. This suggests that the phase-labile component is a persistent tidal rhythm which is following the true rate of tidal progression.

Since approximately a semimonthly period of data is available, an analysis can be made to separate any components of diurnal and lunar frequency in order that their form and phase relationships may be examined. The method is essentially one which has been used for the determination of lunar variations in geophysical data. Its application to the analysis of rhythms in semimonthly periods of metabolic activity in *Uca* has recently been discussed (Webb and Brown, 1961). Briefly, the mean *diurnal,* or *solar daily,* rhythm is obtained by averaging the amount of activity for each hour of the day for the whole 14-day period. Because all phases of the tidal component will have occurred during each hour, their effects upon the mean diurnal rhythm will have been largely randomized. To obtain the mean *lunar daily* rhythm the mean diurnal curve is first subtracted from each of the original 14 days of data to leave the residual lunar component. The hours of the day so treated are next rearranged according to lunar time and averaged. Lunar rearrangement was accomplished by synchronizing for all days the hour on each during which the moon passed through the upper meridian at Belém. The time of upper transit was computed from the American Ephemeris and Nautical Almanac (1962).

As already demonstrated, the actual rate of progression of tidal events varies widely about its mean rate. This variation also occurs with respect to the relatively more uniform rate of progression of the time of upper transit. During the period of study with *U. mordax* the times of high tide ranged from 33 to 120 minutes before the moment of upper transit. Therefore, in synchronizing the times of upper transit one does not perfectly synchronize the times of occurrence of tidal events. This is indicated in Figure 6 where the mean diurnal and lunar components of activity are presented.

The lunar component (Fig. 6B) in motor activity of *U. mordax* resembles the overt tidal rhythm in *U. maracoani* in that maximum activity occurs during the times of low tide, and minimum activity at the times of high tide. *U. mordax,*

FIGURE 5. A. Average daily patterns of spontaneous motor activity in *U. mordax,* from November 24 through December 7, 1962. Triangles indicate times of high tide at Belém where the crabs were collected. B. Daily patterns obtained by combining the mean solar and lunar components of activity in *U. mordax.*

however, differs from *U. maracoani* in possessing a large diurnal component (Fig. 6A) comprised of a broad afternoon maximum and an evening minimum. The range from lowest to highest values of the mean diurnal curve, expressed as percentage of the hour that the crabs are active, is from 10.1 to 21.1, or 11.0. This is slightly exceeded by the range of the lunar variation, which is 12.8.

Because the preceding analysis distinguishes only components of approximately lunar and solar frequency, it is of interest to determine to what extent these components alone can account for day-to-day variations in the patterns of activity in *U. mordax*. For this purpose Figure 5B has been prepared. This figure presents a recombination of the mean solar and lunar variations (see Fig. 6) in their natural phase relationship for each day of the activity study. On Nov. 24, for example, upper transit occurred between 10 and 11 AM. On this day the value for the hour of upper transit in the lunar component was synchronized with the 11 AM value

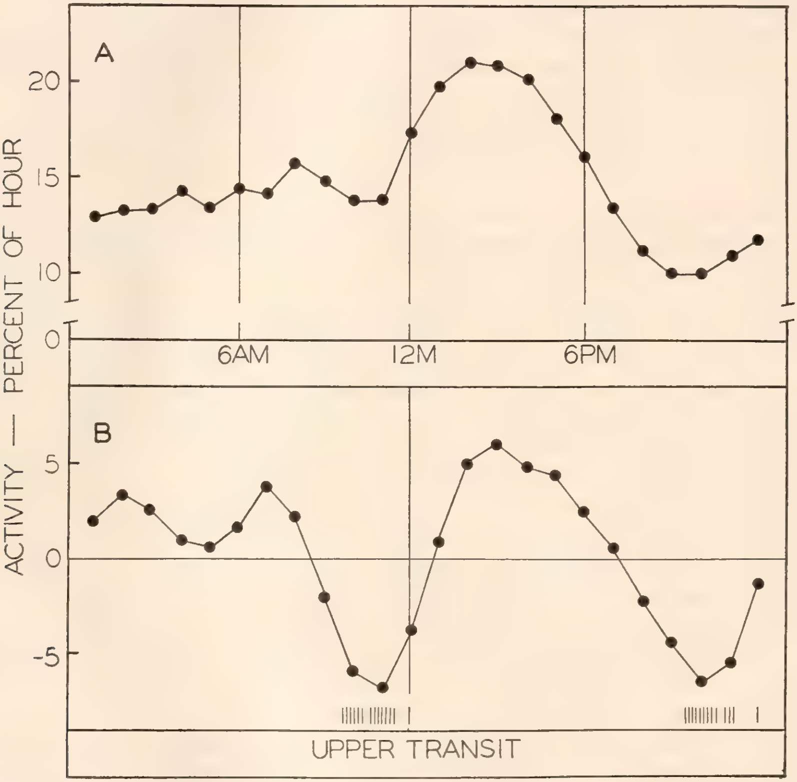

FIGURE 6. A. Mean diurnal component of spontaneous activity in *U. mordax* for the period from November 24 through December 7, 1962. B. Mean lunar component of motor activity in *U. mordax* during the same period. Short vertical lines represent hours of occurrence of incompletely synchronized high tides. Six high tides occurred two hours before upper transit; seven occurred one hour before; and one occurred during the hour of upper transit. Distribution of tides near the time of lower transit is indicated in a similar manner.

for the mean solar component and the two curves were added algebraically. It is thus possible in Figure 5 to compare for each day the pattern of actual recorded activity with the pattern computed from the mean solar and lunar components. More obvious differences between the two sets of curves appear on Nov. 28 through 30 when the relative amplitudes of the morning and afternoon maxima are reversed between actual and computed patterns. Nevertheless, it is clear that comparable major maxima and minima are present at the same times of day for both sets of curves. On Dec. 3 the morning peak in the recorded activity is not conspicuous although its presence is suggested. On Dec. 5 the morning peak of recorded activity appears inverted. On Dec. 6 the maximum occurring over the noon hour is not expressed although the 4 PM peak is present. Aside from these points there is good agreement between the recorded and computed activity. The hour-by-hour correlation between the two sets of data for the entire 14-day period has a coefficient, r, of +0.64. The coefficient of determination, or r^2, is 0.41. Thus 41% of all variation in the activity of *U. mordax* presented in Figure 5A is accounted for in terms of mean diurnal and lunar components. This simple comparison does not take into account the progressive decline in mean level of activity which occurs during the recording period. This decrease involved a reduction of the mean daily activity by almost 50% between the beginning and end of the study. As a consequence the patterns of activity computed from the mean solar and lunar components underestimate the actual level of activity during the first days of recording and overestimate it during the final days. Considerable variation is thereby introduced into the correlation between the two time series and results in a substantial reduction of the coefficient of correlation.

From the foregoing, then, it is evident that the mean lunar and solar components account quite well for major variations in the form of the actual recorded data from which they were statistically derived. Clearly the two components cannot be dismissed as statistical artifacts.

Discussion

Continuous recordings of motor activity or metabolic rate have now been obtained with four species of *Uca*. The persistent rhythm in *U. maracoani* is singular in possessing only an overt tidal component. On the other hand, the rhythm of *U. mordax* resembles rhythms already described for *U. pugnax* and *U. pugilator*. Similarities among the three species are the following. The activity can be resolved into lunar and solar components; the amplitude of the lunar component is equal to, or exceeds, that of the solar; and the major maxima of the lunar component occur near the times of low tide. However, in *U. mordax* the form of the diurnal component differs from that which has been obtained for *U. pugnax* and *U. pugilator* (Brown, 1960), with one exception. In the summer of 1954 the diurnal components in the metabolic rhythms of both *U. pugnax* and *U. pugilator* were essentially inversions of the usual pattern, and they resemble closely the form of the diurnal component of *U. mordax*.

This latter case directs attention to difficulties in attaching ecological significance to the form of rhythmic patterns recorded under laboratory conditions. The atypical form of the 1954 metabolic cycles was subsequently found to be correlated with concurrent inversions of certain geophysical cycles (Brown *et al.*, 1958). Although a number of correlations have been described between geophysical variations and varations in metabolic rate in *Uca* maintained under controlled conditions, it is not known to what extent these have any significance in determining the form or phase of adaptive rhythmic behavior in natural conditions. It has been clearly recognized (Bennett *et al.*, 1957) that activity patterns recorded in the laboratory are influenced by a number of factors including pervasive geophysical forces and, of course, the artificial conditions of the experimental situation.

In connection with the preceding discussion it may be recalled that practically all fiddler crabs have been thought to be diurnal (Crane, 1958), and yet the persistent rhythm of *U. maracoani* collected from a typical habitat shows no conspicuous diurnal component. Even in species with a diurnal component, the persistent tidal cycles of activity are still present during night-time hours. If these crabs are in fact diurnal, this would indicate that the persistent rhythms recorded in the laboratory must be extensively modified in the field by other factors, including possibly the daily cycle of light and darkness. On the other hand, the occurrence and possible extent of nocturnal activity in *Uca* species have recently been discussed (von Hagen, 1962). Such activity has been documented for *U. pugilator* (Burkenroad, 1947) and *U. tangeri* (von Hagen, 1961). A further example is the finding that *U. pugnax* and *U. pugilator* in the Woods Hole area of Massachusetts were out of their burrows and actively feeding when low tide occurred at 2 AM on a moonless night in July (Webb and Barnwell, 1963, unpublished observations).

With *U. maracoani* a closer comparison can be made between the laboratory results and field observations. The behavior patterns of this species have been studied in the field and under near-natural conditions in a "crabbery" by Crane (1958). In the crabbery it was necessary to simulate tidal flooding in order to maintain the normal level of activity and social behavior, including waving and display of the males; but when water level was held constant signs of a tidal rhythm persisted for at least several days. In the light of the laboratory study, this might be expected. However, also present in the field and under tidal conditions in the crabbery were diurnal and semimonthly cycles of social behavior whose timing was largely independent of day-to-day fluctuations in the level of daytime illumination and temperature. These diurnal and semimonthly rhythms were not evident in the present study, in which the tipping pan activity of isolated *U. maracoani* was recorded. Probably the daily rhythm of social behavior was not expressed at all under such conditions, or, even if it were present, it might not be reflected in a recording of spontaneous motor activity.

With the aforementioned reservations in mind it may be suggested that general features of the persistent rhythms in *U. maracoani* and *U. mordax* are related to their respective habitats. *U. maracoani* were collected from burrows on a barren expanse of mud flat near the middle of the tidal range. In this species the persistent activity pattern, an overt tidal rhythm, is correlated with an environment dominated by tidal ebb and flow. *U. mordax* were picked up from the middle and upper levels of drainage ditches along the Rio Guamá, a typical habitat for the species which is the common fiddler crab of tropical rivers and streams of the western Atlantic. This crab has been reported in burrows so far back from the river's edge that it is only reached by the semimonthly spring tides (Crane, 1943). In a species whose activity is less dependent upon the tides, it is not surprising to find a large diurnal component in addition to the tidal component.

The results of experiments with *U. maracoani* and *U. mordax* suggest that the persistent tidal component of their activity rhythms follows the actual variations in the rate of progression of tidal events. This suggestion is based upon correlations between spontaneous changes in frequency of activity cycles and changes in the rate of tidal progression. These correlations occurred during a period when the rate of tidal progression decreased in *U. maracoani* and increased in *U. mordax*. The earlier studies on tidal melanophore rhythms (Fingerman *et al.*, 1958) demonstrated a close adjustment in the phasing of the biological cycle to the tidal cycle. The present activity experiments suggest that there may also be a biological adjustment to the actual systematic variations in tidal frequency.

The hypothesis presented here to account for frequency changes in the tidal rhythms of activity is that the rhythms are timed by the interaction of solar and lunar components. When rhythms of solar and lunar frequency are combined, the solar component tends to displace the times of occurrence of maxima and minima of the lunar component. Consequently, as it progresses across the peaks and troughs of the solar curve, the lunar component appears to deviate around the lunar rate of progression by systematically increasing and decreasing its frequency. In *U. mordax,* variations in frequency of the apparent lunar component are an expected result of the simultaneous occurrence of the solar and lunar components which have been demonstrated for this species. This effect is evident in Figure 5B where the minimum, occurring at about 10 AM on Nov. 24, shifted only two and one-half hours during the first five days but shifted six hours during the next six days from Nov. 29 through Dec. 4. In *U. maracoani* a diurnal component is not expressed. Instead, during each lunar day there are two peaks of approximately equal amplitude whose timing appears to be adjusted to the timing of tidal events at the beach of collection. According to the hypothesis, the pattern of overt behavior would be timed by an underlying solar and lunar interaction. That *U. maracoani* actually does possess the means for measuring accurately a daily period was shown by the persistent daily melanophore rhythm.

This hypothesis does not depend upon whether the timing information which runs the biological clock system is of endogenous or exogenous origin. Possibly the two rhythms of lunar and solar frequency could be produced by an autonomous, internal biological timer. On the other hand, it is equally possible that the organism derives information about the lengths of these natural periods through response to rhythmic geophysical factors in its environment. In the latter case geophysical rhythms would provide information only about frequency and not about phase. Phase would be determined adaptively by factors in the local tidal environment.

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Summary and Conclusions

1. Experimental observations were made on daily and tidal rhythms in four species of fiddler crabs, *Uca,* collected at Belém and Salinópolis near the equator in Brazil.
2. In naturally changing illumination, overt daily rhythms of melanophore activity were present in *U. mordax, U. rapax,* and *U. maracoani.* The form of the rhythms was related to the equatorial cycle of daylight and darkness.
3. There was a melanophore rhythm in *U. thayeri,* but its amplitude was less than one-third of the amplitude of the other species. In *U. thayeri,* as in *U. pugilator,* the melanophores failed to coalesce during the diurnal phase of dispersion.
4. The red chromatophores of *U. rapax* and *U. maracoani* displayed daily rhythms of concentration and dispersion.
5. In constant illumination the amplitude of the melanophore rhythms was reduced. In at least two of the species, *U. mordax* and *U. rapax,* the melanophore rhythm deviated in period from 24 hours.
6. In its spontaneous motor activity *U. maracoani* possessed a tidal rhythm which persisted in constant illumination and temperature. *U. mordax* had a more complex and variable pattern which could be resolved into persistent solar and lunar components.
7. Differences in the activity patterns of the two species appeared to be related adaptively to differences in their habitats.
8. The frequency of the persistent tidal rhythms seemed to follow the actual systematic variations in frequency of the tidal cycle. Such deviations about a true lunar frequency would be expected if the tidal rhythms were timed by two components, one of solar and the other of lunar frequency.

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THE COLLECTING PERFORMANCE OF HONEY BEES UNDER LABORATORY CONDITIONS[1]

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An investigation by Behling (1929) established that the honey bee, *Apis mellifica,* possesses a "Zeitgedächtnis" or time-memory based on the period of the solar or 24-hour day. As is now well known (for a recent discussion, see Renner, 1960), bees, which have been trained to collect sugar water during specific periods of time on several consecutive days, continue to return to the collecting station with greatest frequency at 24-hour intervals when the sugar solution is no longer present. These results obtain when bees are studied under natural environmental conditions or when the animals are maintained in the laboratory under conditions of constant temperature, light intensity and humidity. They indicate, indeed, that the bee has a timing mechanism or biological clock. The functioning of this mechanism is also demonstrated by the time-compensated orientation of honey bees first described by von Frisch (1950).

Studies by Wahl (1932) and newer work by Renner (1955b, 1957, 1959b) confirmed Behling's findings. These investigations also contributed more information regarding this time-memory, and established that this particular indicator of periodicity does share important characteristics with other long-cycle physiological rhythms. Such rhythms have been described for many species of micro-organisms, plants and animals (for recent reviews, see: Annals of The New York Academy of Sciences, 1962; Cloudsley-Thompson, 1961; Cold Spring Harbor Symposia on Quantitative Biology, 1960).

In the course of such investigations, Renner (1955a) developed techniques which can be used to maintain a colony of honey bees for long periods of time in an essentially normal state in the laboratory. Therefore, the investigator has a means of studying indicators of biological rhythmicity of this species throughout the year under constant conditions of light, temperature and humidity. These laboratories have been described and illustrated in detail (Renner, 1955a, 1959a), and the results of studies conducted in them have been published (Renner, 1955b, 1957).

For long-term observation and experimentation, study of the cycle of the return of trained bees to an empty collection station is severely limited. The amplitude of the cycle falls sharply after three or four days (Wahl, 1932). As a

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consequence, the precise identification of the major phase, the time of the greatest frequency of visits to the collecting station, is no longer possible. If a sugar solution is available at all times to bees kept under constant conditions of light, temperature and humidity, will the workers continue to collect during an extended period of time? Is collecting activity then periodic? Does it also indicate the presence of the timing mechanism in this species? These are the questions which the present study was designed to answer.

MATERIALS AND METHODS

Observations of a colony of honey bees which had been living in a bee laboratory for three months while being used for other work suggested that workers do collect sugar solution at varying levels during at least a three-week period under conditions of constant light, temperature and humidity. Because this group had begun to act strangely, it was replaced on February 21, 1961, by a colony which had been kept under natural conditions in the garden of The Institute of Zoology, University of Munich. The temperature of the laboratory in which the bees lived was constant at 27° C., its relative humidity fluctuated non-rhythmically between 48% and 52% and the light intensity during the first three months of the study was constant at approximately 1000 lux, as measured at a level one meter from the floor.

From February 22 until 15:00 on March 23, workers from this hive were marked for identification while collecting a two-molar sucrose solution placed at first in an open vessel on a small table located three meters from the hive. Later, the bees collected the solution from a glass container of 800 cc. capacity which was inside an aluminum chamber (see Renner, 1959b, for photographs of this type of collecting chamber) which, in turn, was on the collecting table. The only manner in which the bees could enter or leave the chamber was through a small (7 by 7 mm.) opening in the side of the chamber. At the underside of this opening was a photoelectric cell whose red-colored beam was broken each time a bee entered or left the aluminum container. This served as the basis of the automatic-recording system which was used. This system has also been described in detail (Renner, 1959b).

The counting and printing apparatus was kept in another laboratory, one floor above the bee laboratory. Consequently, the bees could be left undisturbed for long periods of time. During the first month, an investigator did enter the room every day to mark bees, to observe their collecting, to renew the sugar solution and to remove dead bees. However, during the periods of the recording of activity, the only times a person went into the bee room were at irregular intervals, every five to seven days, when fresh sugar solution was placed in the collecting chamber and dead animals were removed. These procedures were accomplished in most cases in roughly two minutes. The times of these interruptions are indicated by x's in the figures.

The recording of activity, *i.e.*, visits of workers to the collecting chamber, was begun at 15:00 on March 23. At this time it was known that 20 to 25 workers were collecting well. The recording was continued with only two breaks, one on April 15 and one on April 19, until 17:45 on May 2 (Period 1). Both these breaks in the record were caused by minor failures of the printing apparatus.

From the late afternoon of May 2 until 10:00 on May 20, no recordings were made. However, during this interval, sugar solution was available, and the conditions of light, temperature and humidity which had obtained since February 21 were maintained with one exception, on May 19, from 8:00 to 16:10, when the bee room was dark while electrical repairs were made. Also during this interval, the laboratory was cleaned, pollen combs were changed and the bees and their hive were checked for disease or any abnormality.

Recording commenced at 10:00 on May 20. Again, roughly 20 bees were known to be collecting the sugar solution. The activity was recorded until 20:00 on June 18 (Period 2). During this time, 37 hours worth of data were lost because of mechanical difficulties. The environmental conditions were those described earlier. On May 30, the room was again dark from 8:30 until 11:15 because of electrical failure. Further, in an attempt to ascertain the pattern of collecting which occurred after exposure to alternating light and dark periods, 12 hours of dark (18:00 to 6:00) alternated with 12 hours of light (6:00 to 18:00) from 18:00 on June 7 through 6:00 on June 9. After this time, light intensity was constant at 1000 lux.

The level of collecting is expressed by the number of impulses recorded per hour. Since, in general, one impulse is recorded when a bee enters the chamber and a second one when she leaves, the number of impulses equals the number of visits × 2. With the exception of daily averages which are given in Figures 1 through 4, all results have been presented in these figures in terms of the number of impulses per hour. No moving means or averages for several hours have been included.

RESULTS

For Period 1, March 23 through May 2, some collecting activity was recorded for each of the 952 hours for which data were complete. The average level of activity for this period was 225.3 impulses/hour. The minimum, recorded in one case, was 3 impulses/hour, and the maximum, also recorded in one instance, was 559 impulses/hour. During Period 1, the average amount of sugar water collected was 2.50 cc./hour. The level of activity for Period 2, May 20 through June 18, was lower than that for the first period. The average activity for the 648 hours for which complete data were available was 139 impulses/hour. During 20 one-hour periods when the laboratory was light, no activity was recorded. In addition, during those times when the laboratory was dark, there was little or no activity at the collecting station. The maximal activity, recorded for one 60-minute period, was 509 impulses, and the average amount of sugar water collected was 2.20 cc./hour.

Although during Period 1 there was a gradual drop-off in average daily activity from the beginning of recording until its termination, during neither period were there sharp and continuous decreases which might indicate an extremely poor condition of the colony. The daily averages, which are given in Figures 1 through 4, did not indicate any periodic fluctuations when considered against the day of the month.

Figures 1 and 2 present the curves of activity for all days of Period 1 except March 23, the day on which the recording was begun. On this day for 17:00, the

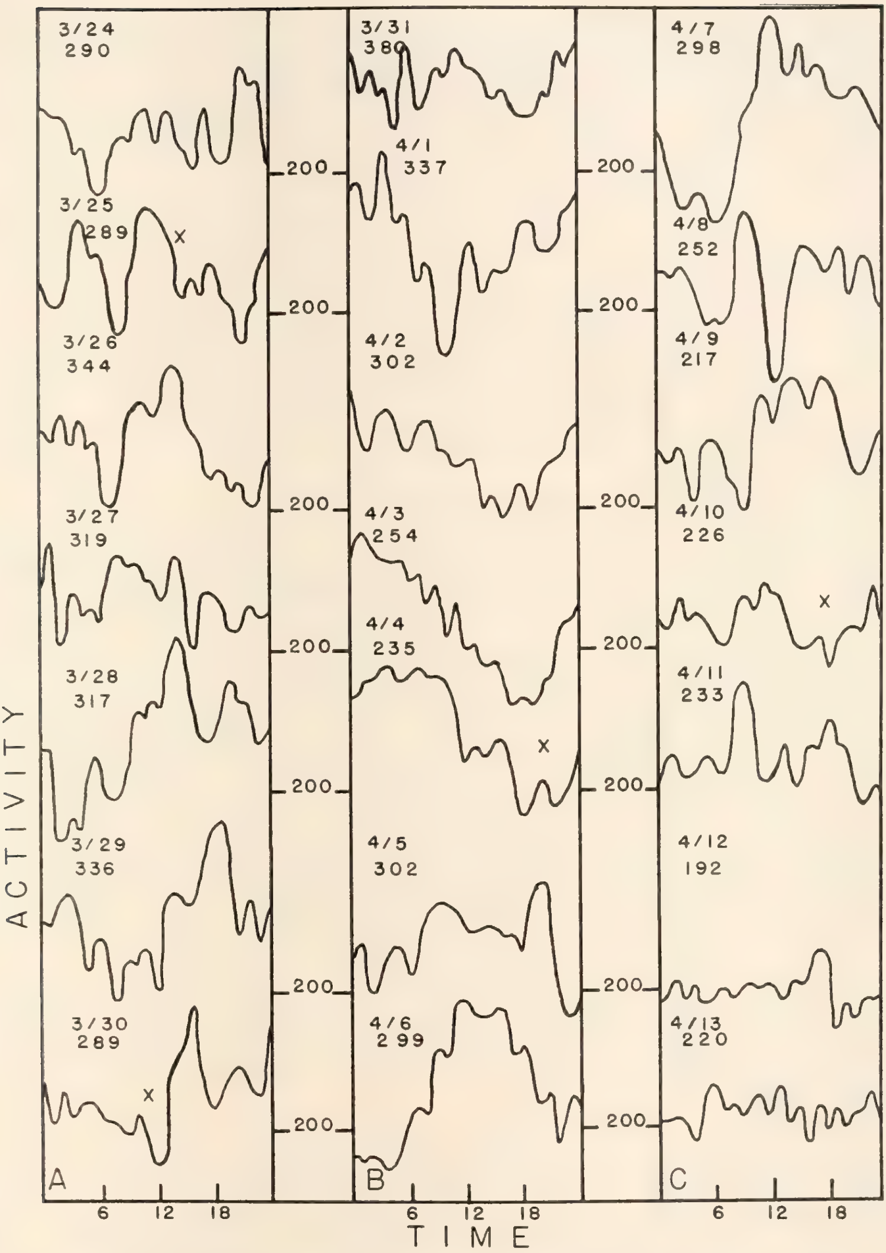

FIGURE 1. The curves of collecting activity for March 24–30 (A), March 31–April 6 (B), and April 7–13 (C). The number of impulses (the 200 level is indicated) per hour is plotted against time in hours, Central European Time. The daily average is given under the respective date. The x's indicate the times at which fresh sucrose solution was placed in the collecting chamber.

first hour for which data were complete, 209 impulses were recorded. Activity then rose to a high of 441 impulses at 21:00 and decreased to 340 impulses at 24:00. During the next 24 hours (Fig. 1, A) activity dropped rather sharply to a low at 6:00, rose again during the middle part of the day, and after lows at 16:00 and 19:00 and a high at 17:00 increased to the maximum at 21:00, after which it decreased. The curve of activity for March 25 was a three-peaked one with collecting having been fairly vigorous at 4:00, over the midday hours and again at midnight. For March 26, the curve was somewhat smoother, and again activity was high during the middle of the day. The picture was generally the same on March 27 and on March 28 when a definite peak for the 24-hour period was recorded at 14:00. Maximal activity on March 29 occurred at 19:00 while generally low activity characterized the late morning and midday hours. This situation was also seen on March 30; however, on that day the peak of collecting occurred at 16:00. The shape of the curve for March 31 (Fig. 1, B) was roughly similar to those for March 26, 27, and 28, except that on this day, the highs at midday and midnight were of the same level, and activity for 6:00 was slightly higher than that at 11:00.

On April 1, a new relationship between the times of maximal and minimal collecting and hours of the solar day was seen. Activity was greatest at 4:00, dropped sharply to a low at 10:00, and then increased fairly smoothly through midnight. The curve for April 2 approaches a sinusoidal one, as do those for April 3 and 4. On April 5, the abrupt increase after 18:00 interrupted the tendency for collecting to fall gradually from the high at 9:00. The curves for April 6 and 7 (Fig. 1, B and C) are among the smoothest recorded during Period 1. In both cases, the maximal activity occurred at noon. That for April 8 resembled the curve for April 7 except that clearly minimal levels of activity at 12:00 and 13:00 were interposed between the late morning and early afternoon periods of greater activity. Again on April 9 collecting was vigorous during the midday with the activity continuing at a high level until 17:00. On April 10, the level of collecting was maximal at 11:00, but the amplitude of this curve is not great. At 9:00 and again at 18:00 on April 11 activity was high, and for April 12 only the maximum at 17:00 and the minimum at 19:00 interrupted an essentially straight line relationship between time and the levels of collecting.

On April 13 and 14 (Fig. 2, A), the amplitudes of the curves were low, while the form of the curve for the 14th was somewhat similar to those for April 2 through 4. For April 15 through 22 (Fig. 2, A and B), collecting was generally lower during the midday hours than it was during the early morning and late evening periods.

Basically, the curves of activity for April 23 through 25 resemble those of April 2 through 4. On April 26 (Fig. 2, C), activity was clearly maximal at 5:00 and was again high at 15:00. During the following solar period, the bees collected relatively actively from 6:00 through 14:00, after which hour the level fell off steeply to a minimum at 18:00. The curve for April 28 is two-peaked with highs at 7:00 and 18:00 and with lows at 1:00 and 13:00. From April 29 through May 1, the temporal patterns of collecting were similar to one another; the daily maxima occurred in each case in the morning hours (6:00 to 8:00), and the daily minima were recorded just after midnight (1:00 to 2:00). On

April 30 and May 1, minor peaks were seen in the afternoon, and for May 2, the data which were available indicated a maximum at 13:00.

The mean period lengths were found for three different blocks of consecutive days (seven to nine days) where some repetition of pattern could be seen. For

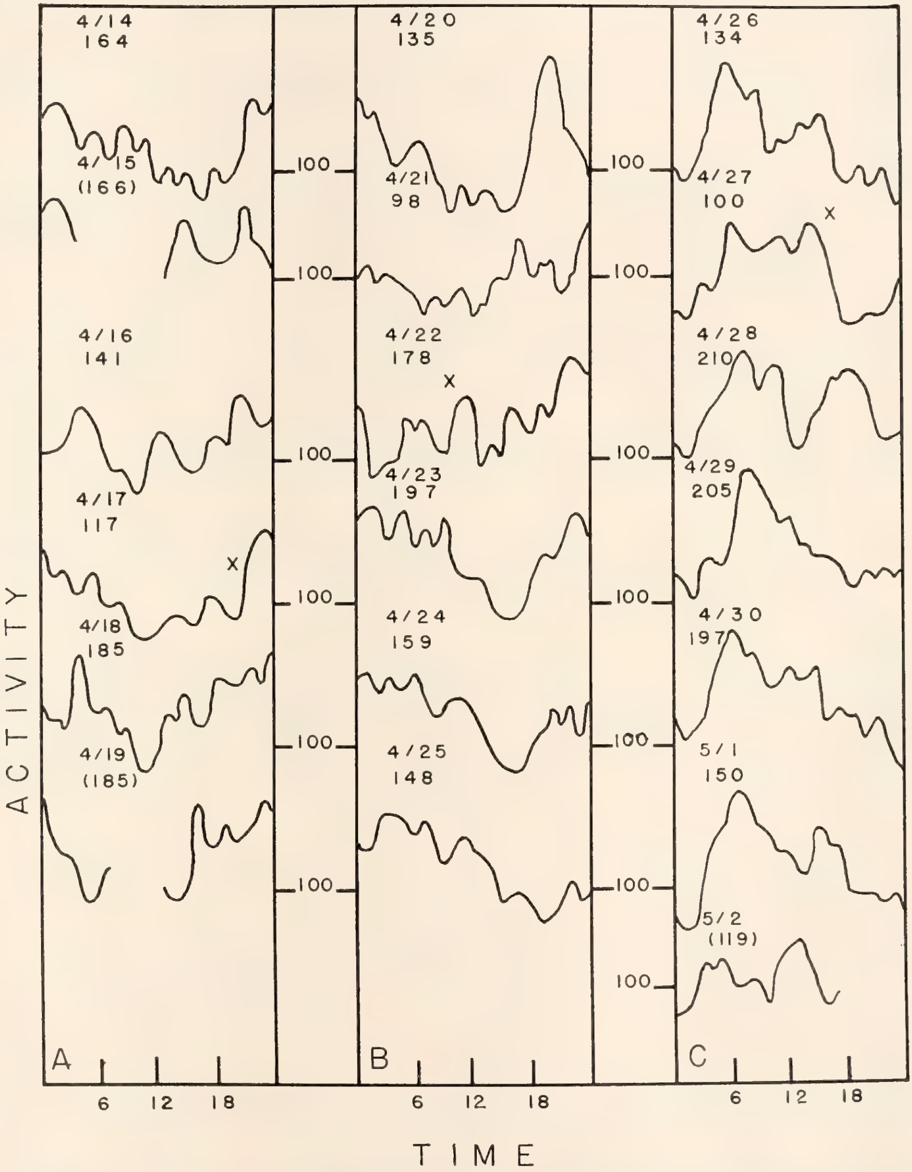

FIGURE 2. The curves of collecting activity for April 14–19 (A), April 20–25 (B), and April 26–May 2 (C). Here the 100 level of impulses is indicated. Remainder of the legend as in Figure 1.

March 25 through 31, the mean interval between highs occurring between 11:00 and 19:00 was 24.0 ± 3.2 hours (range, 19–29); for April 14 through 22, using maxima which fell between 20:00 and 24:00, it was 24.0 ± 2.12 (range, 21–28); and for April 23 through May 1, using maxima which fell between 2:00 and 8:00, the mean was 24.0 ± 1.66 hours (range, 21–26). When the lengths of the periods for these three blocks of days were considered together, the mean was 24.0 ± 2.4 hours.

For single solar day periods, several different forms were described in terms of the level of collecting relative to hours of the day during Period 1. These were:

(1) Relatively high levels of activity occurred bridging midday and midnight periods with intervals of lower levels interposed between them. March 24–27 (Fig. 1, A), March 31 (Fig. 1, B), April 9 (Fig. 1, C), and April 27 (Fig. 2, C).

(2) Collecting was clearly maximal during the midday period. March 28 (Fig. 1, A), and April 6 (Fig. 1, B) and 7 (Fig. 1, C).

(3) Collecting was clearly maximal within a few hours of midnight. This is the inverse of Pattern 2. April 1 (Fig. 1, B), April 17, 18, 19, and 20 (Fig. 2, A and B).

(4) Collecting was high between 0:00 and 12:00, decreased from 12:00, and rose again. April 2 (Fig. 1, B), 3 and 4, and April 23 through 25 (Fig. 2, B).

(5) Collecting activity was maximal roughly midway between 0:00 and 12:00, and was minimal around the midnight hour. April 26, and April 29 through May 1 (Fig. 2, C).

Figures 3 and 4 illustrate the collecting activity for all days of Period 2 except May 20, the day on which recording commenced, and June 12, a day for which no data were available because of mechanical difficulties. As has been pointed out, the laboratory was dark from 8:00 through 16:10 on May 19, the day before recording started at 10:00. From the start until 15:00 on May 20, no collecting occurred; after 15:00 it increased gradually through 24:00, for which hour 157 impulses were registered.

As can be seen in Figure 3, A, on May 21, the level of collecting decreased after midnight, and from 9:00 to 12:00, none was recorded. The activity then increased and remained at a level similar to that of the preceding midnight through the remainder of the day. Again on May 22, the activity of the animals at the collecting station decreased during the early morning hours, virtually ceased from 6:00 until 10:00, increased after 10:00, and was maximal between 14:00 and 16:00. A minor peak was seen at 21:00. The pattern for May 23 was fairly similar to that of May 22 with the exceptions that activity ceased from 4:00 until 8:00; the maximum occurred at 14:00, and the minor peak of the evening was seen at 20:00. The morning hours of May 24 were characterized by a steady increase in activity to the peak at 11:00, while those of the remainder of the day showed decreasing levels of collecting. For May 25, activity increased during the morning hours with highs at 6:00 and from 9:00 to 11:00. During the post-noon period a high at 15:00 and a low at 19:00 were obvious.

The data for May 26 and 27 (Figure 3, B) continued to indicate those tendencies typical of the preceding days. Those for May 27 trace out a fairly smooth curve. The highest point on this day occurred at 10:00 and the lowest at 18:00. On May 28, collecting was low during the entire 24-hour period, and at most, it can be said that the bees were more active from 0:00 through 11:00 than they were during the remainder of the day. The low activity continued through 17:00 on May 29. Collecting then increased rapidly to the peak at 21:00. From then until 10:00 on May 30 the levels of activity dropped fairly regularly. It should be noted that the laboratory was again dark from 8:15 until 11:30 on this day, and after this time, collecting increased until 22:00 after which it began to decrease.

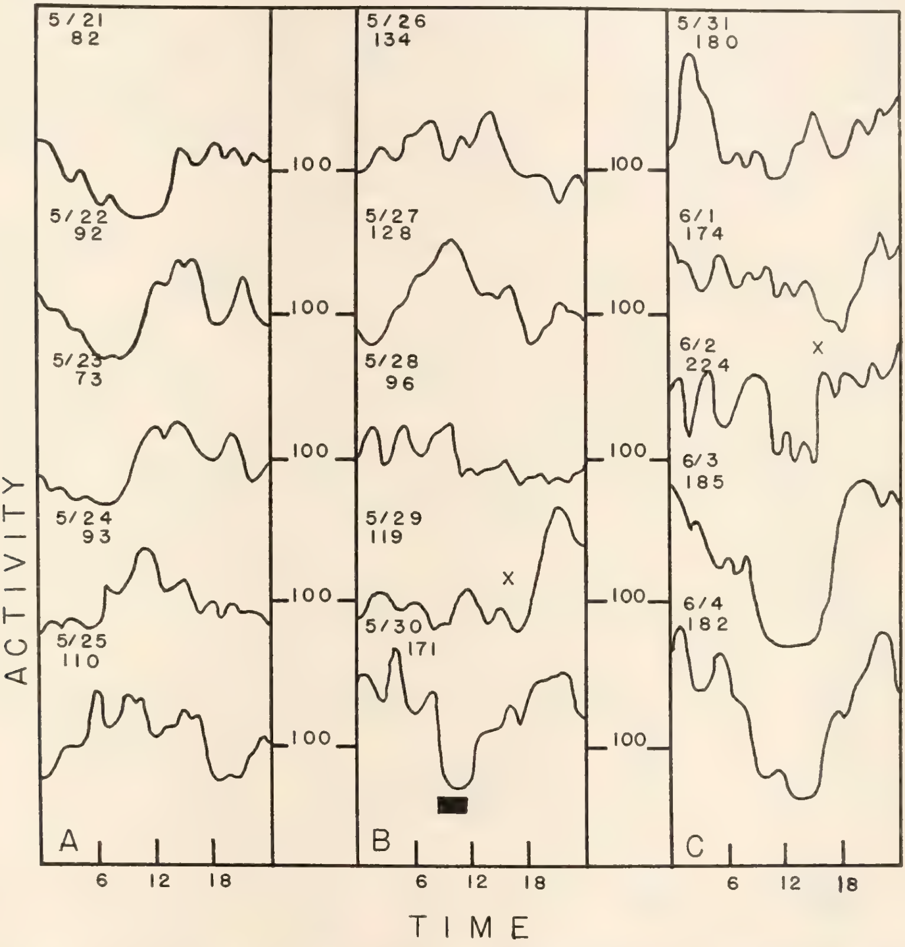

FIGURE 3. The curves of collecting activity for May 21–25 (A), May 26–30 (B), and May 31–June 4 (C). The black band indicates when the laboratory was dark. Remainder of the legend as in Figures 1 and 2.

In general, solar periods characterized by lower activity during midday and greater collecting during early morning and late afternoon and evening hours were seen from May 31 through June 6 (Figs. 3, C and 4, A). The curves for June 3 and 4 were especially smooth. A similar picture was indicated by the data for the first 18 hours of June 7. On this day, as indicated in Figure 4, A, the laboratory was darkened immediately after 18:00. After 6:00 on June 8, at which time the light intensity was again 1000 lux, the bees began to collect. The level of their activity was highest at 14:00 and immediately after that time began to decrease. The laboratory was dark between 18:00 on June 8 and 6:00 on June 9. After this time, light was restored and was then kept constant for the rest of the period of recording. It is important to note that for June 9, the form of the curve is approximately the same as that for June 8 even though, as has been emphasized, the laboratory was not darkened at 18:00.

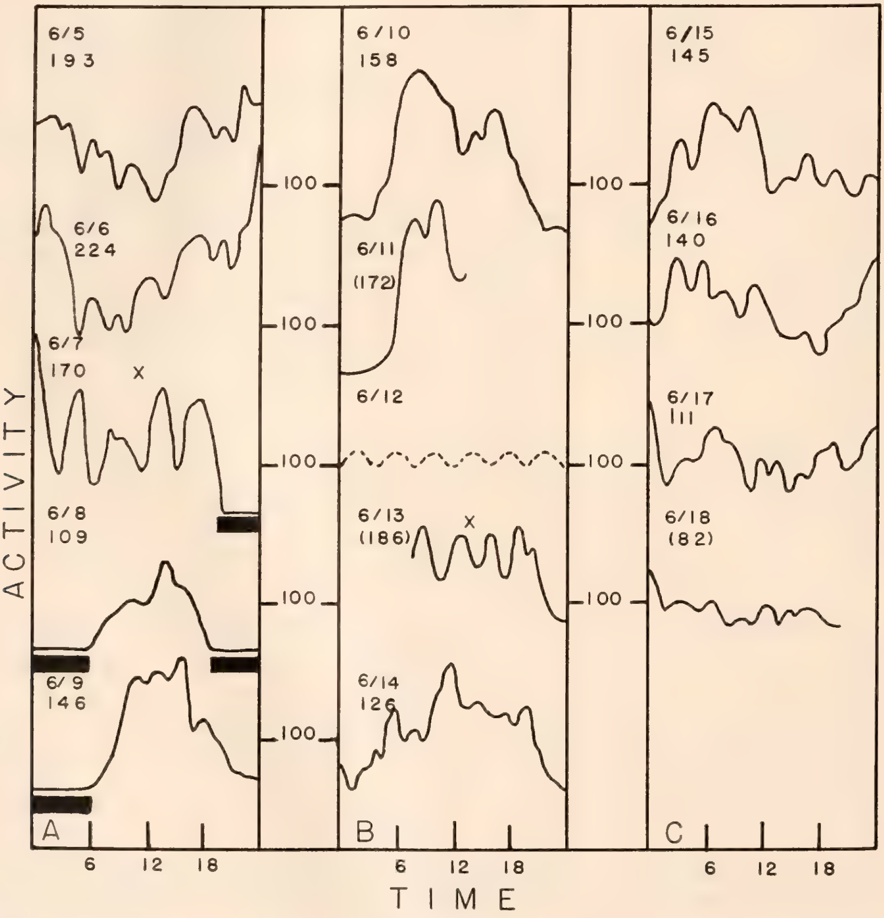

FIGURE 4. The curves of activity for June 5–9 (A), June 10–14 (B), and June 15–18 (C). Remainder of the legend as in Figures 1–3.

This similarity was seen again on June 10 (Fig. 4, B), although the increase in activity started earlier on June 10 than on June 8 and 9, and the results for the periods from 8:00 through 16:00 vary. Unfortunately, no data were available after 14:00 on June 11 until 8:00 on June 13. However, those which were recorded for the first 14 hours of June 11 indicate a pattern which is directly comparable with that for the same hours on June 10. In addition, those data for June 13 after 8:00 showed the tendency for activity to be fairly high until 20:00, after which it fell rather steeply. The curve for June 14 is also very similar to those for June 8 through 10, and in this case the daily maximum occurred at 12:00 and the minimum at 24:00.

Greater activity occurred earlier, between 6:00 and 11:00 on June 15 (Fig. 4, C), than on June 14, and a low was seen immediately after midnight. The period of more vigorous collecting was during the morning hours on June 16. The minimum for this day occurred at 18:00, after which time activity increased. For both June 17 and 18, the curves are of low amplitude. Activity was not great on these days, and only a tendency for midnight collecting to be greater than that of midday was seen.

From the results of Period 2, two blocks of consecutive days were used to calculate mean period lengths. For May 21 through 27 (seven days), by using highs between 10:00 and 18:00, the mean was 23.3 ± 2.60 hours (range 20–27), and for May 31 through June 7 (eight days), by using highs recorded between 0:00 and 4:00, it was 23.7 ± 2.40 (range, 20–28). When these periods are considered together, the mean period length is 23.5 ± 2.45 hours. When these periods from recording Period 1 and from recording Period 2 are used, mean period is 23.8 ± 2.40 hours.

As during Period 1, different forms were seen, but here the variation was less than during the first period, and two principal patterns were obvious.

(1) Collecting was maximal during midday. May 23 through 27 (Fig. 3, A and B) and June 8 through 14 (Fig. 4, A and B). This is comparable to Pattern 2 of Period 1.

(2) Collecting was maximal within a few hours of midnight. May 31 through June 7 (Figs. 3, C and 4, A). This is an approximate inverse of Pattern 1, and is comparable to Pattern 3 of Period 1.

Also to be noted are possible effects of the changing of the sugar solution and the interruption made necessary by this on subsequent activity of the bees. The times when the changes were made are in all cases indicated in the figures. On March 25, the solution was changed shortly after 15:00, and during the next 72 hours, generally high activity occurred at 14:00. A high on March 31 at 11:00 was about 24 hours after the interruption of March 30. The high of April 5 at 20:00 was again approximately 24 hours after the solution was changed on April 4. On April 10, a new cylinder of sucrose solution was presented just after 17:00; on April 11, activity was high at 18:00 and on the following day at 17:00. However, no obvious relationships were evident between the interruption on April 17 or that on April 22 and events on the days which followed. The last change during Period 1 was made shortly after 16:00 on April 27. A peak of activity occurred at 18:00 on April 28.

The first change during Period 2 was made just before 16:00 on May 29. On May 30 at 16:00 and on May 31 at 15:00 minor increases in collecting levels were seen. Nothing comparable was seen on June 3 and 4, the days following the change on June 2 before 15:00. An interruption occurred again after 11:00 on June 7. During the following two solar periods only very minor decreases in the levels of collecting were seen between 11:00 and 12:00. The last change occurred shortly after 13:00 on June 13. A clear maximum was seen at 12:00 on June 14.

Discussion

The results of the present work show clearly that a colony of honey bees, maintained under laboratory conditions of constant light intensity, humidity, temperature and provided with a constant supply of sugar solution, does lend itself to long-term observation and experimentation. Since an investigator need enter the bee laboratory only briefly, at most every five or six days, to renew food supplies and to check the colony and its laboratory, any effects of these manipulations can be kept minimal. During the progress of this study, there was some decrease in the average level of collecting activity. However, this decrease was not constant and is not believed to have been indicative of a poor condition of the colony. This observation was supported by checking the contents of the hive and by keeping a record of the number of individuals that died. The changes in the average daily activity may be explained, in part, by changes in the numbers of active workers.

There was no regular or cyclic variation in these changes. Since data were available for only slightly more than a two-month period, no definite statement concerning the presence or absence of seasonal variations can be made. It is possible that records for a longer period of time would reveal correlations between levels of collecting and seasonal changes. Such correlations have been found for the levels of metabolism of the potato (reviewed by Brown, 1960) and for the metabolic cycles of the fiddler crab (Webb and Brown, 1961). These organisms were also maintained under constant conditions in the laboratory. A colony of honey bees does provide us with a population whose activity should be observed for a year or more under constant conditions.

Not only did the level of collecting of the bees vary from day to day during the two periods of observation, but also, the levels from hour to hour varied. In many cases, the variations were systematic within one solar period, and similar patterns of these variations were maintained from two to nine consecutive days. The mean frequency of these cycles, calculated from five different blocks of days, was 23.8 ± 2.40 hours. These findings suggest that the solar-day timing mechanism known to exist in honey bees is involved in their long-term collecting performance. The reviews of Aschoff (1960) and Pittendrigh (1960) present characteristics of so-called circadian rhythms and theories regarding their organization. Both these authors hold that circadian rhythms are those whose periods under constant conditions *approximate* the period of the earth's rotation, and that these rhythms are endogenous to the living system. The latter author emphasizes the precision of the periods of these cycles during a sequence of days, and states, "Observed standard errors of the period may be less than 2 minutes per day" (Pittendrigh, 1960, Table I, p. 160). Such precision was not manifest in the cycles of the bees. Perhaps a system of recording from which activity per minute or five-minute intervals was available would serve to sharpen these cycles of collecting.

During several solar periods, the differences in the levels of activity were not great and/or varied in a seemingly random manner. Further, in many cases the forms of the cycles changed abruptly from day to day. Therefore, it is illustrated once again that the investigator of biological timing is not confronted with simple cycles to be analyzed solely in terms of period-length. The radical changes seen in the temporal performance of the bees suggest immediately comparisons with results of a series of studies by Brown and his co-workers (see Brown, 1960, for a review of this work). These investigators have described cases in which form, and therefore the period, of cycles of indicator processes do change. At times, these changes are abrupt and irregular from day to day, even when the organisms are maintained under constant conditions including atmospheric pressure. Undoubtedly important to the understanding and elucidation of the mechanism of biological rhythmicity is that aspects of these organismic changes have been correlated with non-cyclic changes which are superimposed on statistically rhythmic changes in the levels of several environmental factors, *e.g.*, cosmic radiation, barometric pressure. Such analyses have not been made with the present data. However, seen here were inversions of the form of the cycles, a phenomenon seen often in the results from Brown's laboratory (Brown, 1957; Terracini and Brown, 1962).

As is well known and is accepted by all students of biological clocks, under so-called constant conditions, the phases of circadian cycles do shift gradually relative to solar time, since the frequency may not be precisely 24 hours (Aschoff, 1960; Brown, 1960; Pittendrigh, 1960). This characteristic has been used by many investigators as proof for the endogenous nature of such cycles (Aschoff, 1960; Pittendrigh, 1960), while it has been explained by another by the theory of autophasing (Brown, 1959, 1960, 1962c). The rate at which such shifts occur, or the length of the free-running period, has been correlated with the constant light intensity to which the organisms are exposed and the habit (diurnal or nocturnal) of the animals in their natural environments (Aschoff, 1960; Pittendrigh, 1960). The performance of the bees does not resemble strictly such changes, for the phases of their activity did not move regularly to a later or earlier time of the solar day or with a constant rate. Rather, often, the relationships of particular phases of activity with solar time changed abruptly and at irregular intervals and in different directions in time. Here it should be noted, as Renner (1955a) found, it is necessary to maintain a very high light intensity (1000 to 1200 lux) in a bee laboratory in order that the bees fly actively. Most forms whose persistent rhythms have been studied have been kept in the dark or at substantially lower light intensities (see Aschoff, 1960, for examples).

Another characteristic of persistent cycles which should be considered here is the temporal lability of phases. Phases can be set relative to specific real times by so-called perturbations of which light-dark and temperature changes seem to be the most effective (Pittendrigh, 1960). Studies of the time-memory of the bee have proved such lability characteristic of this cycle. Phases of vigorous collecting can be set by training at different times or during several different periods of the solar day (Behling, 1929). Exposure to 4.5° C. delays the time of collecting and therefore shifts the phases (Renner, 1957). During Period 1 of the present study, no changes in temperature or light intensity occurred in the laboratory. The effects of change of light during Period 2 will be discussed later. During both periods of recording, the sugar solution was changed at irregular intervals. The observations suggest strongly that the effects of these changes were not extremely great, were not constant, and were ephemeral.

What is the explanation for the saltatory phenomena which have been observed? It is extremely important to point out that in the present work, a *population* of animals was observed. This group is one which has evolved a complex social life that includes a system of communication. This system serves, among other functions, the recruiting of new workers for food collecting (von Frisch, 1946, 1948). One must consider again that individual workers can be trained to forage at particular times, during one or several intervals per solar day, and tend then to return to the collecting station at 24-hour intervals as set by the time of training (Behling, 1929). The data recorded for each day of the present study represent the summation or a complex of the collecting activity of 20 to 30 bees. Is it not probable that the individuals' cycles were not synchronized? Also very probable was that phases of each cycle had been set by a bee's first finding and collecting the sucrose solution, by chance or as a result of having been recruited by active workers. These considerations can explain the different daily patterns which have been seen. On a day when a peak of activity occurred at midday, many of the active workers or the dominant ones were those whose period of most intense collecting had been set at noon. During a day when two periods of high activity were seen, there were either two groups of dominant collectors whose phasing varied or one group which was collecting intensely during two different periods. The maintenance of a pattern during consecutive days, as well as the abrupt changes in form, can be explained, as follows. Workers, whose phases had been set as suggested, were the dominant individuals at the collecting station for several days, and the pattern was repeated. As these died, others whose phasing was different became dominant, and the form of the summated cycle changed. In addition, the possibility that workers were shifting their own cycles, which will be discussed later, must be considered here.

The results of Period 2 certainly illustrate that daily cycles were sharpened by changes in the light intensity in the laboratory. In all probability this increase in precision was the result of the synchronization of the cycles of the collectors. However, fairly regular cycles were not repeated for more than eight days. The high light intensity to which bees must be exposed and its possible effect on the cycles must be considered again as appropriate suggestions for the explanation of the shifts and warpings of the cycles. Possible changes in the dominant workers also can help explain this loss of precision. In addition, it is likely that a worker's phasing is shifted by the changes in light intensity she meets as she flies from a dark hive through an area where light intensity is very high, and into a dark collecting chamber. A glass-walled hive and collecting cylinder might improve this situation. In addition, individuals' performances must be observed during long periods of time to see what the contribution of each to the summated cycle is. A recent paper by Stephens (1962) shows how important it is to consider the contributions of individual fiddler crabs to a rhythm of color change of the population.

Lastly, it is possible that some aspects of the collecting activity of the bees were mediated by external factors whose changes in intensity, rhythmic or not, constitute stimuli for the honey bee. The field study of Renner (1959b) proved that an environmental factor, the position of the sun, when changed by rapid transport of the bees, does warp the form of the cycle of collecting by trained bees. Other possibly effective factors have been considered in two other discussions (Renner, 1960, 1961). It has become increasingly obvious that receptor capacities, and therefore the factors to which organisms can respond, are not known or understood fully. These capacities and reactions may be of utmost importance for the functioning of the organismic time piece and for the future understanding of its mechanism (Brown, 1962a, 1962b, 1962c; Brown, Bennett and Ralph, 1955; Brown, Bennett and Webb, 1960; Webb, Brown and Brett, 1959).

SUMMARY

1. The collecting activity of honey bees, *Apis mellifica,* maintained in a bee laboratory, was recorded automatically through two different periods, the first of 40 consecutive days, and the second of 30 consecutive days. During both these periods, a two-molar sucrose solution was available at all times, and the temperature and humidity were constant. During the first period of recording, the light intensity was also constant at approximately 1000 lux. This was true also of the second period with the exception of four times when the laboratory was dark. Under these conditions, workers continued to collect the sugar solution, and the colony remained in good condition. Therefore, such a population is favorable for long-term observation and experimentation.

2. During both these periods of recording, the average daily collecting activity varied from day to day; however, these variations were not regular or cyclic. The period or frequency of cycles described in terms of hourly levels of activity and solar time was found to vary from 19 to 29 hours, and the mean value was 23.8 ± 2.40 hours. Several different forms were described by these parameters, and changes from one to another were not always gradual, but were saltatory in nature. Changes in light intensity did effect greater precision of the cycles, probably by synchronizing the rhythms of individual workers.

3. The results are discussed in terms of biological rhythmicity, and possible explanations for the saltatory nature of the daily cycles are presented.

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THE GENETICS OF ARTEMIA SALINA. III. EFFECTS OF X-IRRADIATION AND OF FREEZING UPON CYSTS[1]

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One purpose of this study was to find a method of storing brine shrimp cysts without loss of viability. Although Dempster and Hanna (1956) found that they could prevent a gradual decline in hatchability by sealing cysts within a vacuum, their method seemed cumbersome for the small quantities of cysts used in our genetic studies. Another method which appeared promising was that of freezing. Whitaker (1940) reported that cysts could either be maintained in vacuum or subjected to the temperature of liquid air (−190° C.) without affecting the percentage of hatching. However, he did not determine if the nauplii which hatched from frozen cysts could reach maturity. Therefore, experiments were designed in which frozen cysts were tested for both hatchability and ability of the hatched nauplii to reach adulthood.

A second purpose of this study was to find that dose of x-irradiation which would be most efficient for inducing mutations; *i.e.,* the highest dose which would not impair the viability of shrimp emerging from irradiated cysts nor greatly reduce the fertility of inbred stocks derived from them.

The first study of the effects of x-rays on *Artemia* was made by Gajewskaja in 1923 (reviewed by Bonham and Palumbo on pages 155 and 184 of their 1951 paper). Grosch and Sullivan (1955), Grosch and Erdman (1955), and Grosch (1962) showed that when adult shrimp were x-irradiated, 2250 r would sterilize the females but 150,000 r were needed to kill the females. A dose of 200,000 r was required to kill the males. Metalli and Ballardin (1962) also x-irradiated adult shrimps but administered a dose of 1000 r. They found that dominant lethality in the first generation of progeny was much greater for diploid females than for tetraploid females.

Several previous studies have been made of the effects of irradiation upon hatching of *Artemia* cysts: Bonham and Palumbo (1951), Rugh and Clugston (1955), Iwasaki (1958, 1959), Hutchinson and Easter (1960), Easter and Hutchinson (1961), and Engel and Fluke (1962). Only in the study by Bonham and Palumbo was the viability of nauplii observed for a period of more than one week after hatching from x-irradiated cysts. These nauplii were not reared to adulthood. Therefore, this paper will report the results of a study of survival of shrimp hatched from x-irradiated cysts.

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MATERIALS AND METHODS

Source of the cysts

Three collections of dry cysts from bisexual races of *Artemia* were studied. *Sample A* was collected from solar evaporating ponds in San Francisco Bay in the summer of 1960. In the summer of 1961, this sample was divided into ten lots, five of which were frozen. It was obtained through the courtesy of Mr. Maurice Rakowicz, Brine Shrimp Sales Co., Inc., in Hayward, California. *Sample R* was collected from ponds in San Francisco Bay in the summer of 1957. In the spring of 1959 it was divided into two lots, one of which was frozen. It was obtained from the San Francisco Aquarium Society. *Sample U* was collected from Great Salt Lake, Utah, in the summer of 1957. In the fall of 1960 it was divided into two lots, one of which was frozen. It was obtained through the courtesy of Mr. C. C. Sanders, Sanders Brine Shrimp Co., Ogden, Utah.

Irradiation

Cysts from sample A were irradiated in June, 1961. The x-ray source was a General Electric Maxitron 250 operated with a 0.0761 mm. Cu filter (HVL 0.58 mm. Cu) at 250 KeV, 30 ma, at a target distance of 16.5 cm. The dose rate was 3024 r/min. in air. The HVL (half-value layer) was measured with a Victoreen Count Rate Meter and a Victoreen #601 Probe. The output was measured with a Hold #5 chamber and Victoreen R-Meter. The dry cysts were in size 00 gelatin capsules ($\frac{1}{4}''$ outer diameter and $\frac{3}{4}''$ long) at the time of irradiation. Immediately thereafter, the cysts were transferred to dry glass bottles (2.5 ml. capacity).

Storage of cysts

The cyst samples were stored in glass bottles, either in a freezer (−19° to −24° C.) or in darkness at room temperature (20° to 28° C.). The room temperature in our laboratory fluctuates within the narrow range of 21° to 23° C. for about 340 days of each year.

Cyst viability tests

The *Artemia* cyst is actually an encysted blastula, about 200 μ in diameter. When a viable dry cyst is immersed in water, it completes gastrulation. Excystment takes place in two stages. First, the embryo, enclosed within a transparent membrane, emerges from the ruptured shell. A few hours later, it hatches out of the membrane as a free-swimming phototropic nauplius. This process has been described in detail by Whitaker (1940) and by Myint (1956).

The criteria of cyst viability were: (1) *emergence*, (2) *hatching*, and (3) *motility*. Tests were made by counting the number of cysts yielding (1) partially emerged embryos, (2) nauplii free of their enclosing membranes, and (3) nauplii with motile second antennae.

In the earlier methods, *Method A* was used: five cysts were placed in each of 20 shell vials, one-half inch of sea water was added, and the numbers emerged and hatched were recorded. In 1963, *Method B* was adopted. Cysts were scattered on the surface of sea water agar in petri dishes. (This medium was made by adding eight grams of agar to a liter of sea water.) Sterile techniques were not used. The value for each cyst sample was based on data from at least two agar plates. Records were kept on emergence, hatching, and motility. In both methods, counts were made on the second and third days after hydration of the cysts. In method B, the counts could be made more rapidly but hatching and motility values were lower and more variable than in method A. In all experiments reported in this paper, the criterion of cyst viability was hatching when method A was used and emergence when method B was used.

Survival tests

A single layer of cysts was sprinkled onto the bottom of a 250-ml. beaker and 50 ml. of filtered sea water were added. Forty-eight hours later, the nauplii were transferred into the culture medium (50 grams of NaCl per liter of filtered sea water). Two nauplii were placed in each shell vial (21 mm. diameter and 70 mm. high) containing about five ml. of the culture medium. Brewer's yeast was added according to the standard feeding schedule described previously (Bowen, 1962). All survival values were arbitrarily taken to be the number of shrimp alive three weeks after hatching. At this time, all controls had reached at least the twelfth instar of Heath (1924); that is, they could be classified according to sex.

Search for mutations

The shrimp which hatched from the irradiated cysts and four generations of their progeny were examined for mutations throughout the summer and fall of 1961. They were reared in shell vials, fed according to the standard feeding schedule described previously (Bowen, 1962), and were examined without anesthetization under a dissecting microscope (7 ×). All matings consisted of a single pair of shrimp. Pedigree records were maintained on the progeny.

RESULTS AND DISCUSSION

1. *Effect of freezing upon viability*

The three samples, A, R and U, were each divided into two lots. One lot was placed in the freezer while the other was stored in the dark at room temperature. The viability of both lots was tested at intervals. Whenever a sealed bottle was removed from the freezer, it was allowed to reach room temperature before it was opened. This precaution was taken to prevent condensation of moisture upon the cold cysts which might deteriorate with repeated hydration and freezing.

The data in Table I indicate that frozen storage does not impair the hatching percentage of cysts. On the contrary, it prevents the decline in viability associ-

TABLE I

Viability of cysts stored at two different temperatures

Cyst sample	Source of cysts	Length of storage	Viability test method and criterion of viability	Non-frozen storage		Frozen storage	
				Viable/total	%	Viable/total	%
A	San Francisco Bay, California	1 day	A. hatching	332/400	83	304/400	76
		12 days	A. hatching	156/200	78	140/200	70
		22 months	B. emergence	1626/2400	68	1778/2400	74
		25 months	B. emergence	965/1600	60	1223/1600	76
R	San Francisco Bay, California	6 months	A. hatching	887/1790	50		
		13 months	A. hatching	31/60	51	39/60	65
		23 months	A. hatching	19/100	19	63/100	63
		25 months	A. hatching	25/200	12	146/200	73
		4 years	B. emergence	2/1000	0.2	662/1000	66
U	Great Salt Lake, Utah	9 months	A. hatching	32/100	32	63/100	52
		3 years	B. emergence	1/700	0.1	382/700	55

ated with aging at room temperature. In the case of samples R and U, the viability of the unfrozen cysts deteriorated markedly until the percentage of viable cysts was less than 1%. However, in the case of sample A, the viability of the unfrozen cysts did not show a significant decline. This disparity probably can be accounted for by the fact that at the time the three samples were each divided into two lots to be stored at different temperatures, samples A, R and U had aged at room temperature for one, two and three years, respectively. It is possible that decline in viability occurs only after cysts have aged for two years at room temperature. The yearly tests of the viability of these cyst samples will be continued in order to determine if this is the case. The decline in viability of samples R and U seems more extreme than that reported by other authors. For example, Dempster and Hanna (1956) reported that after three years' storage, their control cysts had hatchabilities of about one-fourth to one-sixth of the values obtained

TABLE II

Survival of nauplii which have hatched from cysts stored at two different temperatures. (Survival scored as number of shrimp alive three weeks after hatching. The asterisk indicates that hatchability had fallen so low that adequate numbers of nauplii could not be obtained for a survival test.)

Cyst sample	Source of cysts	Length of storage	Non-frozen storage		Frozen storage	
			Survivors/total	%	Survivors/total	%
R	San Francisco Bay, California	2 years	37/100	37	29/100	29
		4 years	*		70/200	35
A	San Francisco Bay, California	12 days	37/100	37	43/100	43
		22 months	114/200	57	141/200	70

for the initial control sample or of samples stored *in vacuo*. Clegg (1962) obtained 4% hatching from a sample of cysts stored for ten years.

In Table II, it can be seen that freezing of cysts did not impair the viability of the nauplii hatching from these cysts. The only valid comparisons in Table II are comparisons of values in the same horizontal line. This is due to the fact that whenever one cyst sample is tested for nauplius survival, the tests set up at different times yield markedly different results. Additional evidence for this will be seen in Table IV.

In the last experiments on sample R (Tables I and II), the frozen dry cysts had been through four cycles of freezing and thawing. Evidently, this did not damage either the cysts or the shrimp hatching from them. This hardiness may be attributed to the low water content of the cysts (which contain about 8% water removable by vacuum treatment, according to Engel and Fluke, 1962) and their relatively high content of glycerol (about 5%, according to Clegg, 1962).

2. *Effect of x-irradiation upon viability*

Cysts from sample A were divided into ten lots. Two lots were exposed to each of the five levels of irradiation: 0, 400, 2000, 10,000, and 50,000 r. After irradiation, they were transferred into glass bottles and one lot (from each dose) was stored in the dark at room temperature and the other was frozen. Preliminary tests indicated that the 400 r and 2000 r doses did not significantly affect either hatchability of cysts or survival of nauplii. Therefore, subsequent tests were made only of cysts receiving 10 and 50 kr.

In Table III, each value from Experiments 4, 5 and 6 is based on data from two to four agar plates. The difference between control and irradiated values can be assigned to experimental error (discrepancies between plates testing the same lot of cysts). Thus, the 50-kr dose had no significant effect upon cyst viability. This finding is in agreement with the report of Rugh and Clugston (1955) that a 100-kr dose of x-irradiation delivered to dry *Artemia* cysts did not decrease the percentage hatching.

TABLE III

Viability of x-irradiated cysts. (All cysts from sample A *from San Francisco Bay.*

Expt.	Time between irradiation and test for viability	Storage of cysts after irradiation	Viability test method	0 Roentgens		10,000 Roentgens		50,000 Roentgens	
				Viable/total	%	Viable/total	%	Viable/total	%
1	1 day	non-frozen	A	169/200	84	175/200	88	88/100	88
2	13 days	non-frozen	A	217/300	72	215/300	72		
3	52 days	non-frozen	A	164/200	82			127/200	64
4	21 months	non-frozen	B	404/600	67			397/600	66
5	22 months	non-frozen	B	565/800	71	533/800	67	528/800	66
	22 months	frozen	B	618/800	77	621/800	78	539/800	67
6	25 months	non-frozen	B	488/800	61			477/800	60
	25 months	frozen	B	592/800	74			631/800	79

TABLE IV

Survival of nauplii which have hatched from x-irradiated cysts. (All cysts from sample A *from San Francisco Bay. Survival scored as number of shrimp alive three weeks after hatching.)*

Experiment	Time between irradiation and test for survival	Storage of cysts after irradiation	0 Roentgens survivors/total	10,000 Roentgens survivors/total	50,000 Roentgens survivors/total
1	1 day	non-frozen	5/50	11/50	0/50
2	13 days	non-frozen	32/100	29/100	2/100
3	52 days	non-frozen	63/100	73/100	0/100
4	22 months	non-frozen	56/100	58/100	0/100
	22 months	frozen	64/100	77/100	0/100
Total			220/450	248/450	2/450
% Survival			49%	55%	0.4%

Inspection of the data in Table IV reveals the great fluctuation in survival values for nauplii from a single cyst sample when the survival tests are set up at different times. For example, the survival values for non-irradiated nauplii ranged from 10% to 64%. Despite the great fluctuation from one experiment to another, it is clear that, in each of the four experiments, survival of the nauplii from the 50-kr dose was markedly reduced. When the results were totaled, it was found that of the 450 nauplii which hatched from control cysts, 220 reached adulthood and survived to the end of the third week. Of these, 107 were males and 113 were females. Of the 450 nauplii hatching from cysts receiving 10 kr, 248 survived: 128 males and 120 females. In both control and 10-kr groups, the shrimp were 6 to 10 mm. long and all had reached the twelfth instar of Heath (1924). Of the 450 nauplii from the 50-kr group, only two survived: a male and a female. They were the most mature of all the metanauplii in the 50-kr group, yet the male was only 5 mm. and the female only 3.5 mm. in length. Their second antennae were similar to those shown by Heath (1924) to be typical of the eighth and ninth instars for the female and male, respectively. In both shrimp, one eye was larger than the other. The abdomen of the female was constricted and poorly segmented. Both animals failed to mature further and died a few days after the termination of the experiment.

Throughout the survival tests, the shrimp in the 50-kr dose group were smaller and had lower viability than those in both the control and the 10-kr groups. The shrimp in the latter two groups were not significantly different in maturity or viability. The numbers of surviving shrimp are plotted in Figure 1.

It is difficult to compare these survival tests with those of Bonham and Palumbo (1951) because the shrimp in their experiments were maturing at a much slower rate. For example, Table 9 in their paper shows that at the end of eleven days, none of their metanauplii had attained a length of 1 mm. (even in the control group). At this age, all of our controls exceeded that length. Bonham and Palumbo estimated that the LD_{50} for dry *Artemia* cysts was about 50 kr when the experiment was terminated at the end of two weeks (page 185).

If one considers the entire life span of *Artemia*, the data in the present investigation show that the LD_{50} must lie between 10 and 50 kr.

The data in Tables III and IV and in Figure 1 indicate that although the 50-kr dose does not significantly affect the development of the shrimp through the stages of gastrulation and hatching, its effect upon development through the first few instars is extreme. It is surprising to find that a lethal 50-kr dose administered to the encysted blastula does not kill the animal during the earlier stages of gastrulation and embryonic differentiation. This remarkable phenomenon may be explained by the report of Nakanishi *et al.* (1962) that *Artemia* blastulae differentiate without cleavage. These authors found no mitotic figures

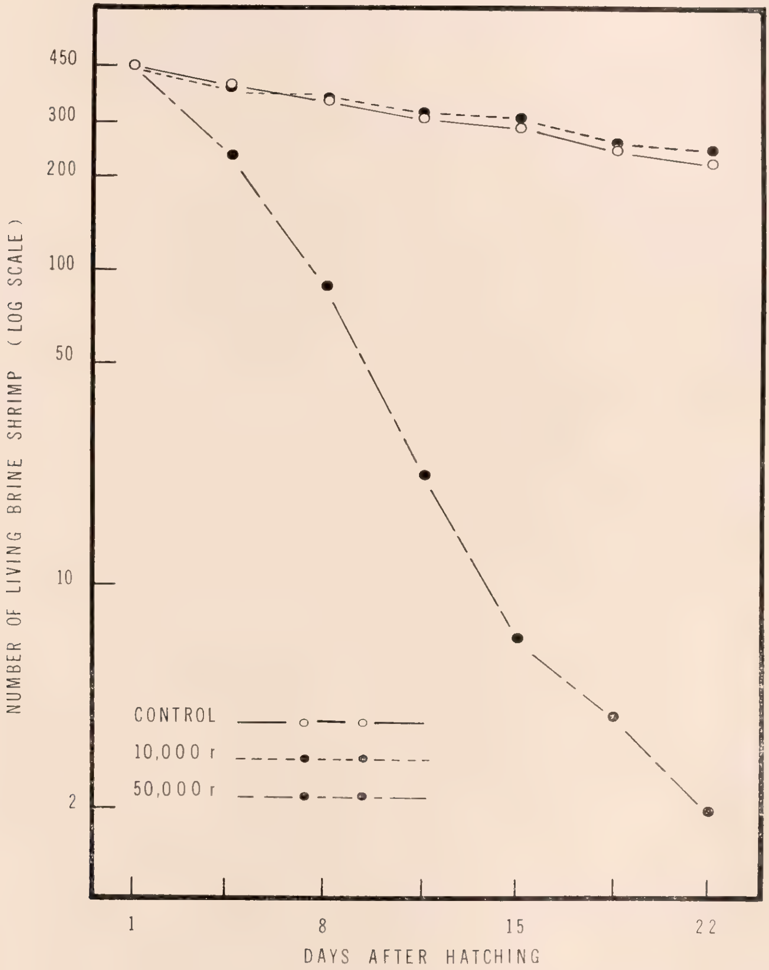

FIGURE 1. Survival of *Artemia* nauplii which have hatched from cysts receiving 0, 10, or 50 kr of x-ray irradiation.

during gastrulation; only a few figures were seen at the time the embryo emerged from the cyst. Post-irradiation cell death rarely occurs until the time that one or several successive mitotic divisions have occurred (reviewed by Lea, 1946; by Puck *et al.,* 1957; by Puck, 1960; and by Jacobson, 1962). For this reason, radiation damage has little chance to express itself in *Artemia* during embryonic development.

The brine shrimp cyst has often been cited as an outstanding example of radioresistance. A small portion of this resistance may be attributed to the fact that tests of emergence or hatching have been used as the indices of radiation damage. However, these tests are made at a time when only a few successive cell divisions have occurred following irradiation.

3. *Search for mutations*

The irradiated shrimp and their progeny were examined in the hope of finding visible (that is, non-lethal) mutations. The abnormal appearance of the two survivors of the 50-kr dose has been described in Part 2. No abnormalities were seen in the shrimp which hatched from cysts given 400, 2000, or 10,000 r doses. Single-pair matings were made up among the survivors of the 2000-r dose and also among the survivors of the 10,000-r dose. These pairs constituted the X-0 generation and their progeny were the X-1 generation. Siblings from the X-1 were mated to produce an X-2 generation. In a similar fashion, sibling matings were continued to produce X-3 and X-4 generations.

Because the dry cysts contain blastulae and because these blastulae differentiate without cleavage (Nakanishi *et al.*, 1962), the nauplii which emerge from irradiated cysts are somatic mosaics. Theoretically, every cell in an emerging nauplius might carry a mutation at a different locus. In adult shrimp of the X-0 generation, dominant mutations would be expressed as patches of abnormal tissue. In the following generations, the shrimp would not be expected to be mosaics. The X-1 generation would show the effects of irradiation-induced dominant mutations. Because females are heterogametic (Bowen, 1963), the X-1 females would show effects of new mutations located on the differential segments of the X and Y chromosomes. It would not be possible to discover mutant traits governed by the irradiation-induced autosomal recessive genes until the X-2 or later generations. Similarly, traits such as the white eye of *Artemia* (Bowen, 1963), which are partially sex-linked, would not be seen until the X-2 or later generations.

The inbred stocks derived from survivors of the 10-kr dose showed considerable loss of fertility which was probably due to x-ray-induced lethals and semilethals. Whenever a deviant shrimp was found in 10 kr progeny, it was outcrossed to the control stock; its siblings and parents were inbred in the hope of producing more progeny with the mutant phenotype. Several aberrant traits were observed in the progeny of shrimp from the 10 kr and 2 kr groups. There were five independent occurrences of absence of setae on the distal lobe of the legs. There were three independent occurrences of swollen abdomen. Other traits were bent abdomen, kidney-shaped eyes, and a bump on the seventh segment of the abdomen. None of these was sufficiently viable for genetic experiments. It is probable that these deformities were induced by irradiation because these departures from the wild-type have not been found in non-irradiated *Artemia*.

A shrimp with garnet eye color appeared in the X-2 generation of the progeny of two shrimp from the 10-kr dose group. It was outcrossed to the control stock and more garnet-eyed offspring were recovered in the F_2. The genetic segregation data have appeared in an unpublished thesis (Hanson, 1963) and will be published in a later paper in this series. Garnet eye color is determined by a recessive autosomal gene. The eye color of homozygous shrimp becomes progressively lighter with age. At three weeks of age, the eyes are garnet (reddish-brown). At six to eight weeks, the eyes are transparent except for a few scattered round cells filled with garnet pigment.

SUMMARY

1. Samples of dry brine shrimp cysts were each divided into two lots: one stored at room temperature and the other stored in a freezer (−19° to −24° C.). Viability of the frozen cysts remained unchanged whereas that of the unfrozen cysts declined. Nauplii hatching from frozen cysts had normal viability. It is evident that frozen storage is superior to unfrozen storage if *Artemia* cysts are to be kept for a period of several years.

2. Dry cysts from a California race were exposed to x-irradiation. Although the 50-kr dose had no significant effect upon the viability of the cysts, it greatly reduced the viability of the nauplii which hatched from these cysts; none of these nauplii were able to live to maturity. It was surprising to find that a 50-kr dose administered to the encysted blastula did not kill during the stages of gastrulation or embryonic differentiation but instead killed during the later developmental stages (the second through the eighth instars). This can probably be explained by the finding of Nakanishi *et al.* that *Artemia* blastulae differentiate without cleavage.

3. The effect of x-rays upon viability was the same when cysts were hydrated and tested immediately after irradiation as when they were tested after two years' storage at room temperature or in a freezer.

4. Irradiated shrimp from the 2-kr and 10-kr doses and four generations of their inbred progeny were examined in hope of finding visible mutant traits. Although many aberrant characteristics were observed, only one viable pure-breeding mutant stock was developed. It is characterized by garnet eye color which is determined by a recessive autosomal gene. The first garnet-eyed shrimp was found in the second generation of the progeny of two shrimp which hatched from cysts given 10 kr of x-irradiation.

5. The 10-kr dose did not impair the viability of cysts or the survival of hatched nauplii to adulthood. It did not completely depress the fertility of inbred progeny derived from the irradiated generation. Therefore, it appears to be an efficient dose for inducing visible mutations for use in genetic studies.

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A CRYSTALLOGRAPHIC STUDY OF VERTEBRATE OTOLITHS

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Although the physiological significance of the static organ is rather well established, little attention has been paid to the structure and composition of the dense bodies in the labyrinth of vertebrates. One exception is the work done on the well-known "ear-stones" of bony fishes. Ever since 1899, when Reibisch demonstrated that fish otoliths could be used for accurate age determinations, these bodies have been subjected to extensive studies. But on the whole, the information available regarding the nature of the calcareous deposits in the labyrinth of vertebrates other than teleosts is very meager.

It has long been known that the dense bodies in the inner ear occur either as solitary large "ear-stones" or as masses of minute particles, "ear-dust." In what follows, the former will be referred to as "statoliths," and the latter as "statoconia." As is customary, the term "otolith" is used in a broader sense to mean any type of a dense body in the labyrinth. It is true that the function of statoliths and statoconia is not exclusively a static one (Lowenstein, 1950), but we prefer the use of these well-known terms to the introduction of entirely new ones.

In the labyrinth of teleosts there are generally three large statoliths, each having an irregular unsymmetrical shape which is characteristic of the species. These bodies derive their names from their shape in carps: sagitta (arrow), lapillus (small stone), and asteriscus (star). They are located in sacculus, utriculus and lagena, respectively. Most other vertebrates, however, have otolith masses consisting of a very great number of small statoconia held more or less firmly together by an organic gel. The statoconia have microscopic dimensions, usually between 1 and 50 microns, are symmetrically shaped and are not characteristic for the species. Recently, otoliths having a size somewhere between statoliths and statoconia have been described by Frizzell and Exline (1958), who suggested the name "ossiculiths" (*ossiculum* bonelet + *lithos* stone) for such bodies. This term is rather unfortunate, not only because of the mixture of Latin and Greek, but especially because these bodies have nothing at all to do with bone. According to these authors, "ossiculiths" should be small (50–500 μ) plano-convex or irregularly shaped particles which are found occasionally with the ordinary statoliths of teleosts. However, in a high percentage of the individuals they occur in one of the labyrinths only, and are then often associated with malformed or abnormal statoliths (Weiler, 1959). "Ossiculiths" can thus hardly be regarded as normal formations in the teleost labyrinth. Irregularly shaped small bodies may, on the other hand, be found regularly together with statoconia, as in the labyrinth of the lamprey, *Lampetra* (Studnička, 1912). In the labyrinth of sturgeons, *Acipenser*, all stages in shape and structure between statoconia and statoliths are found. Some of these intermediate-sized bodies have evidently arisen by the coalescence of several smaller units. "Ossiculiths" are thus not characteristic of teleosts, and a better name and definition of these particles is needed. We suggest here the term "microstatolith" for any endogenous minute body of asymmetric shape found in the vertebrate labyrinth. In addition to the endogenous statoconia, some species of elasmobranchs also have exogenous minute bodies in their labyrinths. These grains, consisting of sea-sand, have entered the inner ear through the endolymphatic duct.

Regarding the composition of otoliths it is commonly stated (even in modern textbooks) that all kinds of vertebrates have otoliths consisting of calcium carbonate in its aragonite form. This is not true. By using x-ray diffraction, Brandenberger and Schintz (1945) found that statoconia of man and domestic fowl consisted of calcite, which is the other well-known polymorph of calcium carbonate. The occurrence of calcite in the labyrinth of a few other species of mammals and birds was later established by Carlström *et al.* (1953), Carlström and Engström (1955), and Sasaki and Miyata (1955). Calcite is also known to constitute the endogenous statoconia of the spined dogfish, *Squalus acanthias* (Vilstrup, 1951). In a crystallographic study of otoliths of 14 vertebrate species, Carlström and Engström (1955) detected still another mineral. The statoconia of the lamprey, *Lampetra fluviatilis,* were found to consist of calcium phosphate instead of calcium carbonate.

The aim of the present study was to make a microscopic and x-ray crystallographic analysis of otoliths from a representative collection of vertebrates. A survey of this kind would unveil the presence of any regularity in the distribution within different vertebrate groups of the different minerals occurring in the labyrinth. If such a regularity were found it would be of value in the classification of certain vertebrates. Moreover, a knowledge of the composition and the physical properties of otoliths should be of interest both from the crystallographic and the physiological point of view.

MATERIAL AND METHODS

The material consisted, if not otherwise stated, of otoliths from fresh adult specimens. After rapid washing in water to get rid of the organic jelly usually embedding the statoconia, the material was dried in air and analyzed as soon as possible. This procedure was necessary since some of the calcium carbonate polymorphs are known to convert quite rapidly to more stable forms, especially under humid conditions. The dried material could be stored for several years without noticeable alterations. The number of vertebrate species investigated was limited to 58. However, these were selected with an aim to cover as far as possible the whole subphylum of vertebrates.

The entire material was analyzed by ordinary powder x-ray diffraction. In cases where both statoliths and statoconia were present simultaneously, they were analyzed separately. The diffraction patterns were recorded with Ni-filtered Cu radiation in a Debye-Scherrer camera having a diameter of 114 mm. In a few instances, micro-diffraction techniques were also employed. Ordinary microscopy, as well as polarization microscopy, was used for the study of ground sections of statoliths and of the size, shape and texture of statoconia.

Here it should be mentioned that even if x-ray diffraction is a supreme method for the identification of crystalline materials, it does not yield any analytical information regarding the nature of non-crystalline materials. In the present study, only the crystalline part of otoliths was studied, and the amount and nature of such constituents as water and organic material were accordingly not determined. Only in one case a completely "amorphous" diffraction pattern was obtained. By adequate treatment of the actual otoliths, the inorganic component could be converted to a crystalline state suitable for x-ray diffraction.

Results

A summary of the results is presented in Figure 5, which also shows schematically the relation between the different vertebrate groups investigated. A detailed description is given below. The vertebrate species are arranged according to the classification of Young (1962).

Superclass Agnatha

Class Cyclostomata

The two still existing orders of this class are represented here by one species each: the lamprey, *Lampetra fluviatilis,* and the hag-fish, *Myxine glutinosa,* both of which have calcareous bodies in their labyrinths. Incidentally, the otoliths are the only calcified structures found in these animals.

In *Lampetra,* the otoliths consisted mainly of statoconia in the form of transparent spheres showing a concentric layering. Their sizes usually ranged from 2 to 25 μ in diameter but were occasionally larger. Quite frequently two or three statoconia were fused together, as illustrated in Figure 1a. In addition a few larger bodies (100–250 μ) were found in each labyrinth. The largest of these had a characteristic, somewhat plano-convex shape and a regular layered structure, as seen in Figure 1b. These microstatoliths were apparently not formed by fusion of a large number of statoconia as suggested by Studnička (1912). X-ray diffraction patterns of statoconia, as well as of the microstatoliths, gave nothing but a broad halo, showing that the mineral salt composing these bodies was in a non-crystalline state. After heating to 700° C., a treatment which causes recrystallization, perfectly sharp patterns of apatite, a basic calcium phosphate, were obtained. Upon solution of fresh otoliths of *Lampetra* in acid, a slight effervescence was observed, indicating that these bodies also contained some carbonate.

In the labyrinth of *Myxine* only statoconia were found. They had the same shape and about the same size as in *Lampetra;* an apatite diffraction pattern was obtained in this case without previous heating. The diffraction lines were, however, very broadened, showing that the degree of crystallinity was fairly low. Since the presence of carbonate hinders the crystallization of apatite, it is inferred that the carbonate content of statoconia of *Myxine* probably is much less than in otoliths of *Lampetra.* This explains why Retzius (1881) did not observe any gas-bubbles upon dissolving in acids the statoconia from *Myxine.*

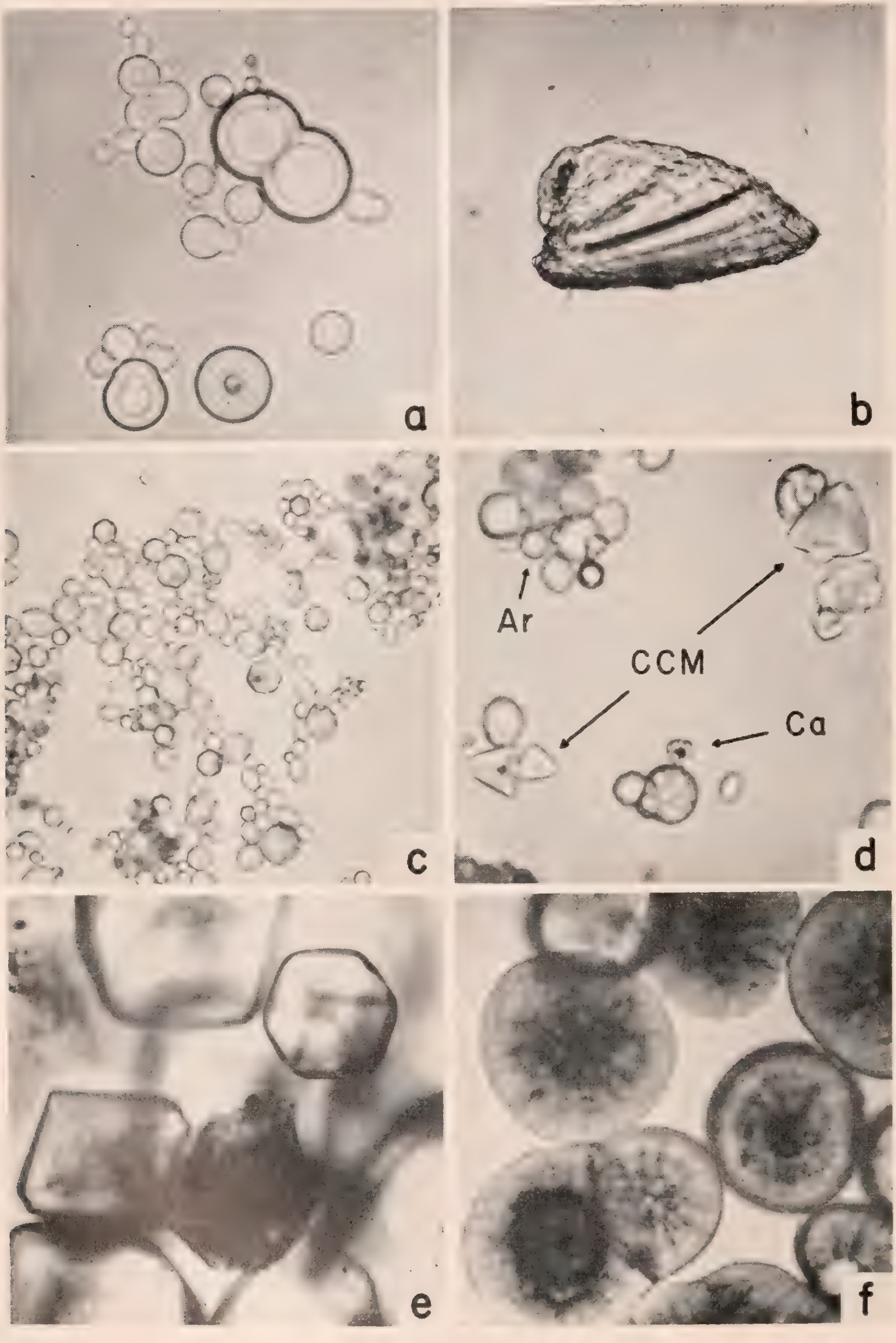
a
b
Ar
CCM
Ca
c
d
e
f

FIGURE 1.

Superclass Gnathostomata

Class Elasmobranchii

In the subclass Selachii, the otoliths of the following 9 species, including 6 sharks and three rays, were investigated: the porbeagle, *Lamna cornubica;* the bull-shark, *Carcharhinus leucas;* the lemon-shark, *Negaprion brevirostris;* the nurse-shark, *Ginglymostoma cirrhatum;* the tiger-shark, *Galeocerdo cuvier;* the spined dog-fish, *Squalus acanthias;* the starry ray, *Raja radiata;* the thornback ray, *Raja clavata;* and the common skate, *Raja batis.* The four first-mentioned sharks and the three rays had statoconia of a rather similar appearance. Usually, they were lemon-shaped, having a rounded or slightly hexagonal cross-section; almost spherical or quite elongated statoconia were also frequently observed. A typical example is shown in Figure 1c. The size varied from a few up to about 40 microns, with a mean size of roughly 10 μ. In polarized light, the statoconia were highly birefringent but each statoconium did not show perfect extinction, indicating that they did not consist of single crystals. This was also evident from the x-ray diffraction analyses. Diffraction patterns of stationary specimens gave no "spottiness" of the diffraction lines, in spite of the relatively large particle size, which means that each statoconium was a heavily distorted single crystal or rather a polycrystalline body. In all these cases, the diffraction patterns showed the statoconia to consist of pure aragonite. In the case of the tiger-shark, on the other hand, the microscopic investigation showed that besides statoconia of the type described above, two additional crystalline materials were present. One type, which was less frequent, consisted of small irregularly shaped particles apparently made up by clusters of spherites, while the other type, which constituted about one third of the total number of statoconia, formed perfect single crystals having the shape of trigonal bipyramids. The latter were also highly birefringent and ranged in size from a few up to 20 microns. In addition to aragonite lines, the powder diffraction pattern of statoconia from the tiger-shark showed faint calcite lines and fairly strong lines of a substance which could not be recognized as any known mineral deposit of biological origin. In diffraction patterns of stationary specimens, the lines from this unknown substance were easily distinguished from the aragonite and calcite lines because of their "spottiness." Hence, they were caused by the trigonal single crystals observed microscopically.

From the x-ray diffraction pattern, the trigonal crystals could be identified as calcium carbonate monohydrate ($CaCO_3 \cdot H_2O$), a substance which to our knowledge never has been encountered in nature before. A photomicrograph of statoconia from a tiger-shark, showing the three different calcium carbonates present, is shown in Figure 1d.

The statoconia of the spiny dog-fish, on the other hand, showed no similarities

FIGURE 1. Otoliths of cyclostomes and elasmobranchs: Statoconia (a) and a microstatolith (b) of the lamprey, *Lampetra.* Figures (c)–(f) show statoconia of the thornback ray, *Raja;* the tiger-shark, *Galeocerdo;* the spined dog-fish, *Squalus;* and the rat-fish, *Chimaera,* respectively. In (d) three different minerals can be distinguished: Ar = aragonite, Ca = calcite, CCM = calcium carbonate monohydrate. Note the dark quartz grain in (e) among rhombic calcite crystals. Magnification: (a) and (c)–(f) 750 ×, (b) 200 ×.

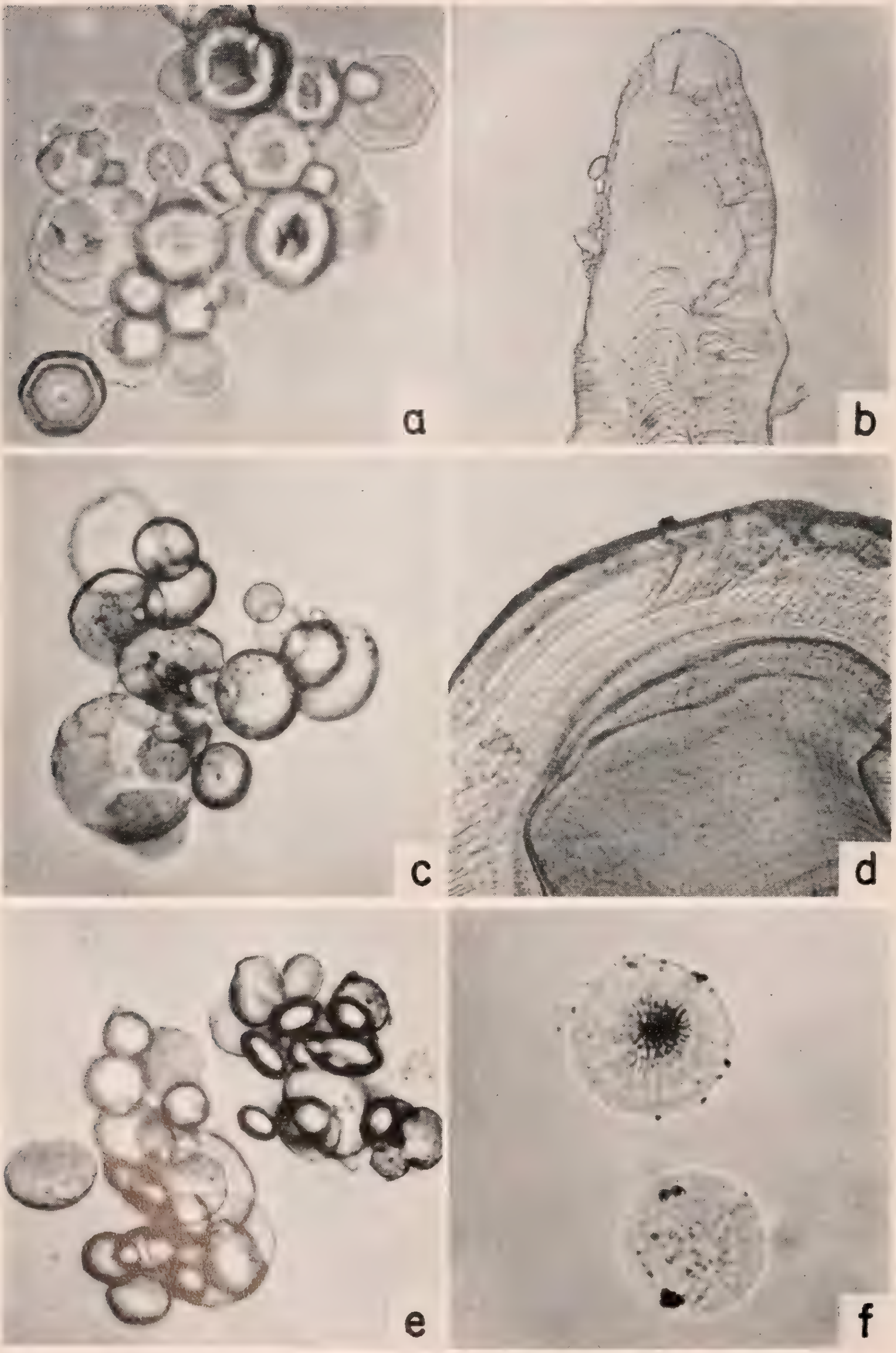

Figure 2.

to those already described. Three specimens investigated all had statoconia of both endogenous and exogenous origin, but the relative amounts of the two types varied in different specimens. The endogenous statoconia were quite large (some 30–150 μ), forming water-clear rhombohedral single crystals which, from the x-ray diffraction analysis, were found to consist of pure calcite (Fig. 1e). The exogenous grains, on the other hand, had very irregular shapes and varied from colorless and transparent to black and opaque. Since they were insoluble in weak acids, they could easily be separated from the endogenous calcite statoconia. X-ray diffraction patterns of samples treated in this manner showed the exogenous bodies to consist mainly of α-quartz (SiO_2) plus minor amounts of ilmenite ($FeTiO_3$), *i.e.*, sea-sand. It is well-known that some elasmobranchs, presumably those having a wide ductus endolymphaticus, may use sea-sand as statoconia. Thus, Retzius (1881) observed irregular grains in the labyrinth of *Acanthias;* and Stewart (1903–1906) seems to have been the first to identify these particles as sand. Exogenous statoconia are also known to occur in *Pristiophorus japonicus* (Iseltöger, 1941), *Rhina squatina* (Stewart, 1903–1906; Nishio, 1926), *Torpedo ocellata* (Nishio, 1926; Werner, 1930), and *Torpedo marmorata* (Werner, 1930). While some authors state that the statoconia of these species consist of nothing but sand, others have found both sand and endogenous statoconia or only endogenous statoconia in the same species. This discrepancy may be due partly to varying proportions of the two constituents in different specimens, but it may also depend on the use of preserved material in which the calcite particles may have disappeared. In the case of *Rhina squatina*, however, it is possible that this shark depends entirely on statoconia made up by foreign particles. When investigating a mature embryo of this species, Stewart found no statoconia at all, while in an unborn *Squalus acanthias* used for comparison there was an abundance of endogenous statoconia. Whether preserved or fresh material was used by Stewart is not known, and his observation certainly needs confirmation.

In the subclass of Bradyodonti, the rat-fish, *Chimaera monstrosa*, was investigated. Its statoconia consisted of almost perfect spherites of a brownish tint showing a concentric lamellar structure as well as a radial striation. Their diameter was quite large (20–100 μ), as seen in Figure 1f. The x-ray diffraction patterns showed them to consist of pure aragonite. Such spherites of aragonite were not found in any other vertebrate species during this investigation, but they are probably not unique for *Chimaera*. According to Nishio (1926), the statoconia of the shark, *Heptanchus cinereus*, are concentrically layered spheric bodies of about the same size as were here found in *Chimaera*. In *Heptanchus*, these statoconia are reported to be cemented together by an amorphous calcareous material, thereby virtually forming a kind of statolith.

Class Actinopterygii

In the superorder Chondrostei, the otoliths of two sturgeons, *Acipenser sturio* and *Acipenser güldenstädti*, and of two species of bichir, *Polypterus ornatipinnis*

FIGURE 2. Otoliths of Chondrostei and Holostei: Statoconia (a) and a ground section of a statolith (b) of a sturgeon, *Acipenser*. Figures (c) and (e) show statoconia from two species of bichir, *Polypterus delhezei* and *P. ornatipinnis*. A ground section of a statolith from the former in (d). Statoconia of the bow-fin, *Amia*, in (f). Magnification: Statoliths 200 ×, statoconia 750 ×.

and *Polypterus delhezei,* were investigated. In accordance with the findings of Retzius (1881), Shepherd (1914), and Weiler (1959), both statoconia and statoliths were found in the labyrinth of *Acipenser sturio.* This feature seems to be general in sturgeons, since it here was found also in *Acipenser güldenstädti* and is reported in *Acipenser ruthenus* (Weiler, 1959). In addition, microstatoliths—*i.e.*, small irregularly shaped bodies—were found, in accordance with the observation by Weiler (1959). The statoconia showed quite a variation in size, from a few up to 100 microns. The smaller statoconia generally were of a rounded shape with an indication of a hexagonal outline, whereas the larger ones were more disk-shaped. In these latter, concentric hexagonal lamellae were observed (Fig. 2a). The optical examination, as well as the x-ray diffraction, showed that the statoconia in spite of their regular polyhedral shape were not perfect single crystals. The extinction position was, however, fairly sharp, and good conoscopic interference figures could be obtained, indicating that the sub-units building up the statoconia must be well aligned. The statoliths, on the other hand, were definitely polycrystalline and had the usual lamellar structure found in statoliths of teleosts (Fig. 2b). The x-ray diffraction patterns showed that all types of bodies found in the labyrinth of *Acipenser sturio* consisted of pure vaterite, the third and most unstable modification of anhydrous calcium carbonate. In the case of *Acipenser güldenstädti* the diffraction patterns, besides the dominating vaterite patterns, showed faint lines of aragonite and calcite. However, this specimen had been preserved in formalin for several months and the presence of these two minerals was probably an artifact. Both the aragonite and the calcite might have been produced by transformation of vaterite.

The two *Polypterus* species here investigated both had well-developed statoliths. Regarding the fine structure, the statoliths resembled completely those of teleosts. However, in both species small amounts of statoconia were also found in the labyrinth, side by side with the statoliths, a feature which seems to have been overlooked by earlier investigators. The statoconia were lens-shaped with a diameter of about 5–40 microns and showed no evident internal structure. Photomicrographs of a thin section of a statolith, as well as of statoconia, are shown in Figures 2c, d, and e. The x-ray crystallographic examination showed that while the statoliths were composed of pure aragonite, the statoconia consisted of pure vaterite.

In the superorder Holostei two species, the bow-fin, *Amia calva*, and the garpike, *Lepisosteus osseus*, were investigated. The otoliths of the latter came from a whole dried head. The static bodies of both species very much resembled those of *Polypterus*, *i.e.*, they consisted of statoliths of the ordinary teleost type as well as of thin, lens-shaped statoconia often showing a faint radiating structure. The composition of these otoliths was the same as in the case of *Polypterus*, with statoliths consisting of pure aragonite and statoconia consisting of pure vaterite.

In the superorder Teleostei the otoliths of 13 species were investigated. The material consisted of herring, *Clupea harengus;* salmon, *Salmo salar;* pike, *Esox lucius;* catfish, *Corydoras aeneus;* common eel, *Anguilla anguilla;* needlefish, *Belone belone;* platy, *Xiphophorus maculatus;* cod, *Gadus callarias;* haddock, *Gadus aeglefinus;* bearded cottus, *Agonus cataphractus;* perch, *Perca fluviatilis;* sole, *Solea solea*, and angler, *Lophius piscatorius.* As could be expected, only statoliths were found in all species. In the vast literature on the static bodies of teleosts, there seems to be only one exception to the common rule that teleosts possess three well-defined and characteristic bodies in each labyrinth. According to Thompson (1888) the sun-fish, *Mola mola,* should possess statoconia instead of statoliths.

The microscopic structure of the teleost statoliths is well-known and needs no elaboration here. After the careful investigation by Irie (1960), using microradiography, electron microscopy, and x-ray diffraction, there is not much to add regarding the structure of these polycrystalline bodies.

One of the first chemical analyses of teleost statoliths was carried out by Wicke (1863) who found that the statoliths of cod consisted of 91.1% inorganic and 8.9% organic material. The inorganic salt was found to be calcium carbonate admixed with small amounts of magnesium carbonate, calcium phosphate and calcium sulphate. On the basis of optical data, Immermann (1908) suggested that the calcium carbonate should be in its aragonite modification, a proposal which has been fully confirmed by later investigations. In the present material no other crystalline component than aragonite was found. This is in perfect accordance with the few earlier x-ray crystallographic investigations made on teleost statoliths. Brandenberger and Schintz (1945), Sasaki and Miyata (1955), and Irie (1955) investigated the statoliths of *Esox lucius, Scomber scombrus* and *Pseudosciaena manchurica,* respectively. Aragonite was found to be the only crystalline material in these cases. The statement by Lunde (1929) that statoliths of cod, in addition to $CaCO_3$, water and organic material, should also contain 11.44% CaO therefore seems quite surprising. It is not only improbable that calcium oxide should occur in a non-crystalline state, thereby escaping detection with x-ray diffraction, but it is quite impossible for this compound to form and to remain stable in the presence of water. Not only are the statoliths surrounded by an aqueous medium *in vivo* but they also contain some water at crystal interfaces and in the organic phase.

Occasionally, abnormal statoliths are found in teleost labyrinths. The structure and composition of these bodies was not investigated but it seems probable that the calcium carbonate in abnormal statoliths may occur in another modification than the ordinary aragonite. This is not yet proven, but at least in one case reported by Immermann (1908) an abnormal statolith of plaice, *Pleuronectes platessa,* seemed to be composed of calcite.

Class Crossopterygii

This class is represented here by the only living coelacanth, *Latimeria chalumnae,* and by one dipnoid, the Congo lung-fish, *Protopterus dolloi.* The *Latimeria* material consisted of the right sagitta from two individuals, the second and the seventeenth specimens caught. Because of the rarity of this material a more detailed description will be given here. The statolith of the second *Latimeria* was quite large, forming an almost circular, concavo-convex plate with a rounded edge. The average diameter was about 21 mm. and the average thickness about 4 mm. The convex surface was rather smooth while the slightly concave surface showed concentric lamellae, as well as a rough tubercular central part (Figure 3a and b). The weight was 2683 mg. and the density 2.775. A microradiogram showed internal cracks radiating from the center. Since these cracks were not

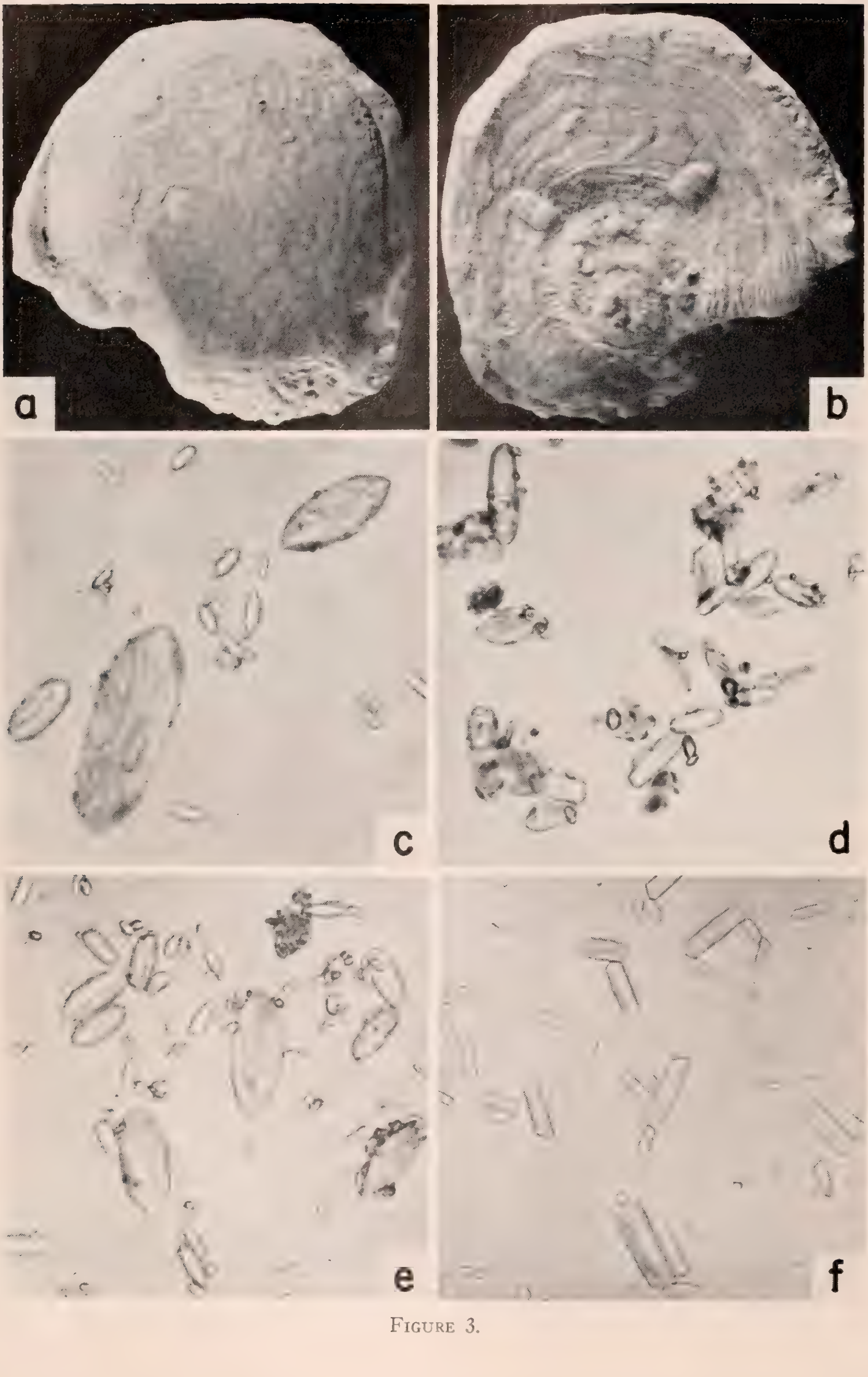

FIGURE 3.

filled by the immersion liquid used for the density determination, the figure obtained was certainly a trifle too low. In the uneven central area a few very small and water-clear crystals having well-developed faces were observed. Since the statolith had been lent on the condition that it should not be destroyed, material for the x-ray diffraction analysis was taken from the surface and from a depth of 1 mm. through a 0.5-mm.-wide drill hole. The small transparent single crystals were found to consist of pure calcite, whereas the sub-surface sample, as well as material taken from different sites on the surface, consisted of pure aragonite. The statolith of the seventeenth *Latimeria* was very similar to that already described. Its average diameter was slightly less, around 19 mm., but it was strikingly thinner, resulting in a weight of only 1428 mg. The central tubercular area of this statolith was quite pronounced and consisted to a large extent of calcite. The rest of the statolith was apparently made up of aragonite with the exception of a thin yellowish crust covering large parts of the surface. This crust proved to consist of apatite which most probably had been deposited during the preservation. The presence of calcite in both specimens might or might not have been produced *in vitro* but the finding of small amounts of mineral other than aragonite does not interfere with the over-all picture of the *Latimeria* statolith as being of the typical teleost type. According to Anthony (personal communication), the labyrinth of *Latimeria* seems not to contain any other dense bodies than the single, large sagitta. However, detailed information on the anatomy of the inner ear of *Latimeria* is still missing and we do not therefore know if, besides the statolith, there are also statoconia present, as is the case in Chondrostei and Holostei.

In the labyrinth of the lung-fish, *Protopterus dolloi*, only statoconia were found. They were spindle-shaped with pointed ends (Fig. 3c), having lengths varying from about 1 micron or less up to 100 microns. It is well established that all Dipnoi have statoconia in their labyrinths (Retzius, 1881). According to Weiler (1959), *Neoceratodus* and *Protopterus* also possess microstatoliths. While Shepherd (1914) found statoconia in *Lepidosiren paradoxa*, he claims that the static bodies of *Neoceratodus forsteri* consist of two statoliths, namely, lapillus and sagitta, but he remarks (p. 106) that they "are very chalky-looking." This finding is certainly a misinterpretation, possibly due to the use of preserved instead of fresh material.

Optical as well as x-ray diffraction examination showed that, in contrast to the finding in all other fishes, the statoconia of *Protopterus* consisted of perfect single crystals of aragonite. Such statoconia are otherwise only encountered in the classes of Amphibia and Reptilia.

Class Amphibia

No member of the subclass Apoda was examined.

In the subclass Urodela, three species were investigated, namely the European salamander, *Salamandra maculosa*; the smooth newt, *Triturus vulgaris*, and the

FIGURE 3. Otoliths of Crossopterygii and amphibians: Statolith of *Latimeria* seen from its convex (a) and concave (b) side. Statoconia of the Congo lung-fish, *Protopterus* (c); the European salamander, *Salamandra* (d); the clawed toad, *Xenopus* (e); and the common frog, *Rana* (f). Magnification: (a) and (b) 3.6 ×, (c)–(f) 750 ×.

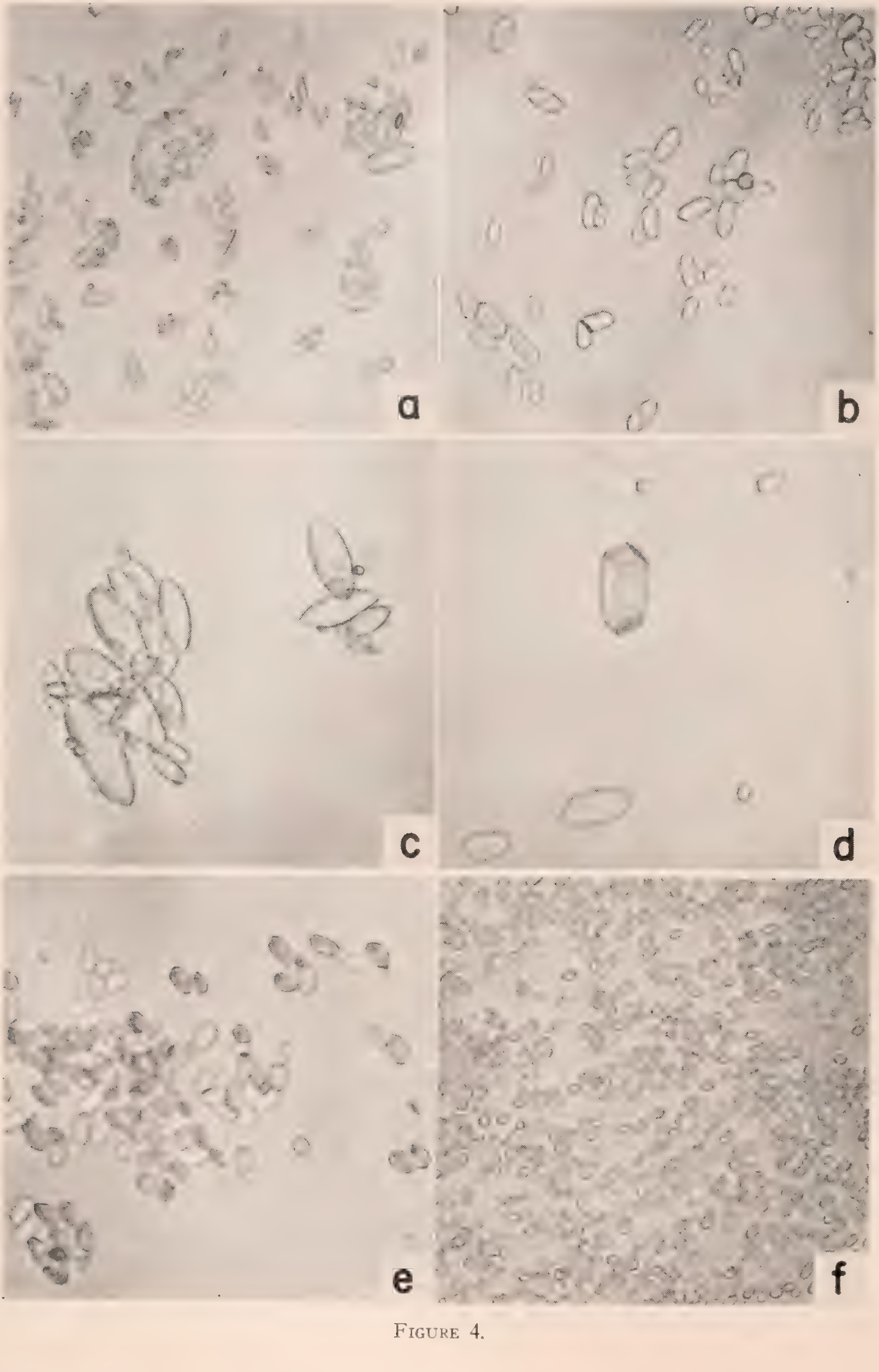

FIGURE 4.

axolotl, *Amblystoma mexicanum*. Of the last mentioned species, only formalin-fixed material was available. In all cases the statoconia were elongated particles with pointed ends having lengths of 3–40 μ (Fig. 3d). They consisted of single crystals of pure aragonite. This seems to be in conflict with the finding of Hastings (1935), who from x-ray diffraction as well as chemical analyses reported the occurrence of some 20% of calcium phosphate (apatite), together with aragonite, in otoliths of *Amblystoma tigrinum*. The explanation of this observation is that the axolotl has a most unusual kind of otholith, consisting of densely packed statoconia surrounded by a thin, almost transparent shell. Diffraction patterns of this shell during the present investigation showed it to consist of poorly crystalline apatite. It is not known whether such composite "statoliths" occur in other larval urodeles, but Sarasin and Sarasin (1890), examining the species *Ichthyophis glutinosus* belonging to the subclass Apoda, found that the embryos possessed a single rounded statolith in each labyrinth whereas adult specimens had the usual arrangement found in Amphibia, *i.e.*, a great number of statoconia. The statoliths of the embryos may possibly have had the same structure as larval *Amblystoma*. Further investigations on urodeles are needed to clarify the function of the composed "statoliths" of the larval stage and their relation to the statoconia of adult animals.

In the subclass Anura, the material came from the following species: the common toad, *Bufo bufo;* the common frog, *Rana temporaria*, and the clawed toad, *Xenopus laevis*. In all these cases, the labyrinth bodies, as well as the content in the endolymphatic sacs, consisted of elongated single aragonite crystals having tapered ends. The statoconia of *Rana* had well-developed crystal faces while the statoconia of *Bufo* and *Xenopus* were more spindle-shaped (Figs. 3e and f) and resembled the statoconia of the Congo lung-fish (Fig. 3c).

The otoliths of frogs have been analyzed earlier by means of x-ray diffraction. In accordance with the above findings, pure aragonite patterns have always been obtained (Funaoka and Toyota, 1928; Brandenberger and Schintz, 1945; Carlström and Engström, 1955; Sasaki and Miyata, 1955).

Class Reptilia

The subclass Anapsida was represented by two species: a European tortoise, *Testudo* sp., and the European water-tortoise, *Emys orbicularis*. In both, the labyrinth contained statoconia and these were rounded or elongated. While the statoconia of *Testudo* had an average size of about 10 microns, those of *Emys* were smaller, the average being only about 5 microns. The x-ray diffraction patterns showed typical aragonite diagrams but, in addition, rather strong calcite lines were present. The aragonite-to-calcite ratio was estimated to 3:1.

In the subclass Lepidosauria, the static bodies of four species were analyzed: the slow-worm, *Anguis fragilis*, a monitor lizard, *Varanus flavus*, the grass-snake, *Natrix natrix*, and the adder, *Vipera berus*. All species possessed statoconia of about the same size and shape: elongated single crystals with pointed ends having

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FIGURE 4. Otoliths of reptiles, birds and mammals: Statoconia of the common grass snake, *Natrix* (a); pigeon, *Columba* (b); the carpercaillie, *Tetrao* (c); the opossum, *Didelphis* (d); the porpoise, *Phocaena* (e); and sheep, *Ovis* (f). Magnification 750 ×.

lengths of some 1–50 microns (Fig. 4a). The diffraction patterns showed aragonite diagrams on which faint calcite lines were superimposed, except in *Varanus* in which the statoconia gave a perfectly pure aragonite pattern.

No fresh material was available from the subclass Archosauria.

Class Aves

In the subclass Neornithes, containing the three recent superorders Paleognathae, Impennae, and Neognathae, only the last mentioned was represented in this investigation. The static bodies of the following species were analyzed: pigeon, *Columba domestica;* domestic fowl, *Gallus domesticus;* the capercaillie, *Tetrao urogallus;* and the magpie, *Pica pica.* These species had statoconia of the usual form, *viz.,* elongated particles with pointed ends having an average length of about 10–20 microns. The x-ray crystallographic analysis yielded diagrams of pure calcite and the microscopic examination showed the statoconia to consist of perfect single crystals.

From an optical examination, Herzog (1925) claimed that the statoconia of domestic fowl consisted of aragonite. However, by means of x-ray diffraction, Brandenberger and Schintz (1945) could later show that they consisted of calcite.

Class Mammalia

No member of the subclass Prototheria was available. In the subclass Theria, on the other hand, both the infraclasses Metatheria and Eutheria were repre-

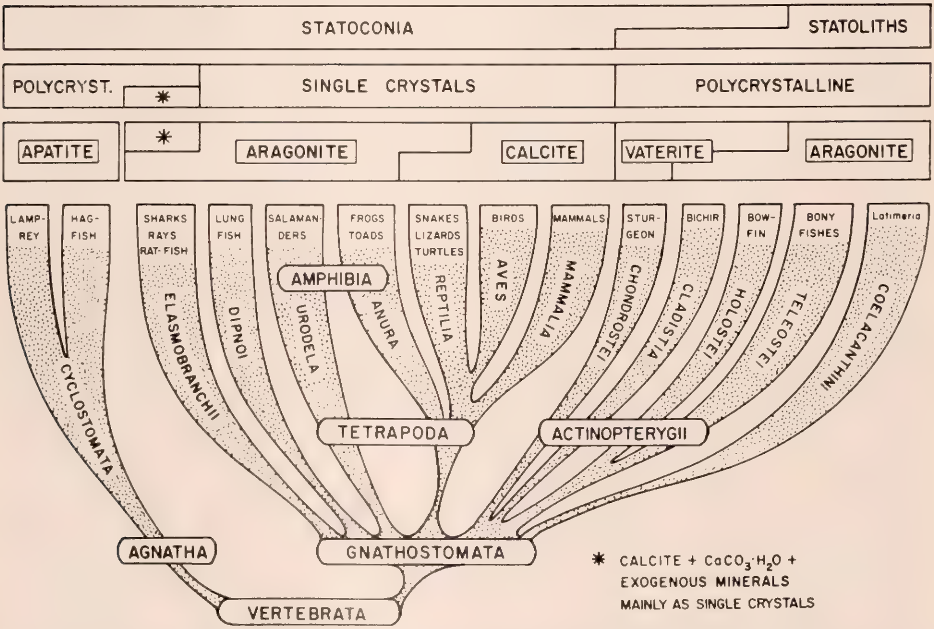
STATOCONIA
STATOLITHS
POLYCRYST.
✱
SINGLE CRYSTALS
POLYCRYSTALLINE
APATITE
✱
ARAGONITE
CALCITE
VATERITE
ARAGONITE
LAMP-REY
HAG-FISH
SHARKS RAYS RAT-FISH
LUNG FISH
SALAMAN-DERS
FROGS TOADS
SNAKES LIZARDS TURTLES
BIRDS
MAMMALS
STUR-GEON
BICHIR
BOW-FIN
BONY FISHES
Latimeria
CYCLOSTOMATA
ELASMOBRANCHII
DIPNOI
AMPHIBIA
URODELA
ANURA
REPTILIA
AVES
MAMMALIA
CHONDROSTEI
CLADISTIA
HOLOSTEI
TELEOSTEI
COELACANTHINI
TETRAPODA
ACTINOPTERYGII
AGNATHA
GNATHOSTOMATA
VERTEBRATA
✱ CALCITE + $CaCO_3 \cdot H_2O$ +
EXOGENOUS MINERALS
MAINLY AS SINGLE CRYSTALS

FIGURE 5. A schematic drawing showing the present knowledge regarding the distribution of statoliths and/or statoconia, their texture and mineral composition throughout the vertebrate series.

sented. The material from the former consisted of otoliths from the opossum, *Didelphis marsupialis*, while the material from the latter came from: the common shrew, *Sorex araneus;* man, *Homo sapiens;* guinea-pig, *Cavia porcellus;* rabbit, *Oryctolagus cuniculus;* cat, *Felis domesticus;* the gray seal, *Halichaerus grypus;* sheep, *Ovis aries;* and the porpoise, *Phocaena phocaena.*

All mammals investigated possessed elongated statoconia with pointed ends (Fig. 4d–f). They usually were quite small, rarely exceeding 10 microns in length; but in the labyrinth of the seal and the opossum, particles up to 20 μ in length, were occasionally observed. The latter species also had much larger particles consisting of an intergrowth of several statoconia. The statoconia were found to be single crystals. In all cases, they provided x-ray diffraction patterns of pure calcite. This finding is in agreement with earlier x-ray crystallographic analyses on statoconia of *Homo* carried out by Brandenberger and Schintz (1945), Carlström *et al.* (1953), and Sasaki and Miyata (1955). Besides human statoconia, the latter authors also investigated statoconia from guinea-pig, rabbit and rat. In all cases calcite patterns were obtained.

Discussion

A. *Crystallographic aspects*

In the present study, two essentially different calcium salts, calcium phosphate and calcium carbonate, were found to compose the otoliths of vertebrates.

Calcium phosphate was found only in cyclostomes, where it existed in a non-crystalline or poorly crystalline state. In the latter case it had an apatite structure. Apatite is a mineral which is rarely encountered in calcareous deposits of invertebrates, but it is the most common mineral of vertebrates, since it is the main constituent of their teeth and bones. Biogenic apatite is usually a slightly impure hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$. In vertebrate skeletons it is always cryptocrystalline, *i.e.*, the apatite crystals are of colloidal size. The main "impurity" is carbonate, usually 1–5% CO_2. However, the carbonate does not form a separate calcium carbonate phase but is somehow associated with the apatite. There seems to be a proportionality between the carbonate content and the degree of crystallinity. From *in vitro* experiments it has been shown that the presence of carbonate disturbs the crystallization of apatite (Trautz, 1960). In the present material it was not possible to determine the CO_2 content of the cyclostome otoliths because of the small amount of material available. However, from a comparison with the degree of crystallinity of biogenic apatites with known carbonate content, it is inferred that the statoconia of *Myxine* should contain less than 5% CO_2, whereas the carbonate content of the otoliths of *Lampetra* probably is in the order of 10%.

Calcium carbonate was found to constitute the statoliths and statoconia of all other vertebrates investigated, that is, all gnathostomes. Calcium carbonate is extremely common as skeletal material in invertebrates but is not reported to occur in vertebrates except in otoliths, in egg-shells of birds, and in some pathological deposits. Anhydrous calcium carbonate ($CaCO_3$) is known to crystallize in three different polymorphs, namely, as calcite, aragonite, and vaterite. While calcite belongs to the rhombohedral system, both aragonite and vaterite crystallize in the orthorhombic system. Vaterite, however, is pseudohexagonal. In all three polymorphs the planar CO_3-groups have their planes mutually parallel, an arrangement which explains the very high birefringence of these carbonates.

Under ordinary conditions calcite is the stable form. Although aragonite is metastable, its transformation to calcite is usually very slow. Vaterite, on the other hand, is quite unstable, especially when in contact with water; when dry it may stay unaltered for several years. All the calcium carbonate polymorphs can be artificially produced and a mixture of the three is usually obtained *in vitro* when precipitated from concentrated solutions. Calcite is often the predominating one and is also most easily obtained in pure form. It is well-known that elevated temperature (30–70° C.), as well as the presence of small amounts of Mg^{+2}, Sr^{+2}, Ba^{+2}, Pb^{+2} or SO_4^{-2}, favor the formation of aragonite. Low temperatures, on the other hand, seem to favor the precipitation of vaterite, but this polymorph is rather difficult to get totally free from contamination by the other two. The chemical and physical conditions which regulate the formation of the different $CaCO_3$ polymorphs *in vitro* seem not to be known in detail. Since the physico-chemical conditions of the endolymph are even less known, it is not possible to say why one or the other calcium carbonate polymorph is produced *in vivo*. It is, however, interesting to note that the calcium carbonate modifications of otoliths usually are in an extremely pure state. Only in a few instances was a mixture of two polymorphs encountered in the same labyrinth. It is of special interest that two different crystal forms of $CaCO_3$ can exist in a perfectly pure state side by side, one forming the statoliths and the other forming the statoconia. This was the situation in the labyrinths of *Polypterus, Lepisosteus,* and *Amia.*

Regarding the occurrence of the three calcium carbonate polymorphs in nature, calcite is by far the most common. Not only is it a rock-forming mineral, but it also constitutes the inorganic salt of the skeletons of many invertebrates, such as Foraminifera, echinoderms and crustaceans (Chave, 1945). Aragonite, although quite rare as a mineral, is also common in biogenic deposits; scleractinian and massive octocorals and hydrocorals, for example, have skeletons consisting of extremely pure aragonite (Chave, 1954; Wainwright, personal communication). A mixture of the two is also common: mollusc shells and pearls are built up in layers of calcite and aragonite crystals (Wilbur, 1960). The third $CaCO_3$ polymorph, vaterite, seems, on the other hand, to be exceedingly rare in nature. It is reported to occur, together with calcite and aragonite, in the repair tissue of gastropod shells (Mayer, 1931) as well as in pathological calcifications such as urinary calculi and bile stones (Lagergren, 1962). To our knowledge, however, it has never before been found as the only crystalline component in a calcareous deposit. The occurrence of pure vaterite in the labyrinth of some fishes indicates the presence of a component stabilizing vaterite *in vivo*.

Besides the polymorphs already mentioned, which are usually well-crystallized in biogenic products, calcium carbonate may also occur in a non-crystalline state. In analogy with the situation for calcium phosphate, the crystallization of calcium carbonate is disturbed by presence of phosphate, resulting in amorphous or poorly crystallized material. Such non-crystalline deposits, mainly consisting of calcium carbonate, are known to occur in biological calcifications. Thus gastroliths, as well as large parts of the carapaces of many crustaceans, consist of amorphous calcium carbonate admixed with small amounts of phosphate. Whether this type of calcium carbonate is hydrated seems not to be known. From the present investigation there is no indication that non-crystalline $CaCO_3$ occurs in otoliths. It may, however, be a transitory step during their formation.

The finding in the present study of a well-crystallized hydrated calcium carbonate, $CaCO_3 \cdot H_2O$, as a biogenic product is certainly unique. This calcium carbonate was until recently unknown. In 1950, Brooks *et al.* succeeded for the first time in identifying the monohydrate which they prepared synthetically. A detailed description of its crystallographic properties is given by Lippmann (1959), who synthesized single crystals up to 0.1 mm. in size. Since this compound may be encountered in other biogenic deposits consisting of calcium carbonate, its x-ray diffraction characteristics are given in Table I. The unit cell dimensions found (a = 6.100 Å, c = 7.553 Å) are in good agreement with those given by Brooks *et al.* (1950) : a = 6.15 Å, c = 7.61 Å, and by Lippmann (1959) :

TABLE I

Powder diffraction data of biogenic $CaCO_3 \cdot H_2O$

Indexing on a hexagonal pseudocell (a = 6.100 Å, c = 7.553 Å) to d-values not smaller than 1.75 Å. Relative intensities: vs = very strong, s = strong, ms = medium strong, m = medium, w = weak, vw = very weak. Reflections with smaller d-values than those listed are all w or vw.

Index (hkl)	Spacing in Å		Intensity	Index (hkl)	Spacing in Å		Intensity
	$d_{calc.}$	$d_{obs.}$			$d_{calc.}$	$d_{obs.}$	
001	7.55	—		112	2.373	2.372	w
100	5.29	5.30	w	103	2.273	2.271	vw
101	4.33	4.34	vs	202	2.164	2.165	ms
002	3.78	—		210	1.997	1.997	w
102	3.07			113	1.942	1.942	w
		3.07	s				
110	3.05			211	1.930	1.929	s
111	2.827	2.829	m	004	1.889	—	
200	2.641	—		203	1.823	1.823	w
003	2.517			104	1.778	1.778	w
		2.510	vw				
210	2.493			212	1.765	1.765	w

a = 6.13 Å, c = 7.54 Å. The small differences observed are probably due to slightly different composition of the $CaCO_3 \cdot H_2O$ samples investigated. The above dimensions, according to Lippmann, relate to a hexagonal pseudocell. The true unit cell has an a-axis = $a\sqrt{3}$.

It should also be pointed out that the external shape of the single crystals of aragonite and calcite occurring as statoconia in land-vertebrates cannot serve for the identification of these minerals. Irrespective of the nature of the mineral, the single-crystal statoconia are almost invariably spindle-shaped. If crystal faces are developed they are usually too poor for a precise identification. In fact, the erroneous statement by Herzog (1925) that the statoconia of domestic fowl consist of aragonite was based on measurements of the angles between crystal faces.

B. *Biological aspects*

Since some striking differences in the occurrence and crystallographic properties of statoconia and/or statoliths were found between different groups of vertebrates (see Fig. 5), the possible biological significance of these differences needs a closer consideration. Our suggestions cannot, however, be regarded as having a general validity until more extensive material has been examined. It is fully realized that chemical relationships *per se* have a rather restricted value. The very same and highly specialized compound may be found in biological material of vastly different origin. To take an example, the respiratory blood pigment, haemoglobin, consisting of haeme-groups linked to polypeptide chains (which may vary in composition), is common to all vertebrates. However, it is also found in some annelids and crustaceans and in one or two species of insects and molluscs. In addition, it has been detected in a few plants (Fox, 1949). In the case of the vertebrate labyrinth, however, the situation is somewhat different. Not only are the otoliths homologous formations but their physiological role is almost identical throughout the vertebrate series. The sensitivity to gravitational force and linear acceleration is indeed a very primitive property in the animal kingdom. Moreover, these forces remain the same irrespective of the medium surrounding the animal. It can be inferred, therefore, that the otolith organ, when once developed, should be unaffected by such alterations in the outer environment as the step from an aquatic to a terrestrial life.

The anatomical division of vertebrates into two main groups, the Agnatha and the Gnathostomata, is reflected in the composition of their otoliths. Two chemically different minerals were found in the two groups, suggesting entirely different mechanisms during the formation of their static bodies.

When looking at the systematic distribution of the various calcium carbonates deposited in the labyrinth of gnathostomes, a general trend is discernible. Thus, aragonite and vaterite are found exclusively in cold-blooded vertebrates, whereas all warm-blooded vertebrates have otoliths consisting of calcite. Calcite was also found in the labyrinth of two sharks; the possible reason for this will be considered below. In reptiles, which form the bridge between warm- and cold-blooded vertebrates, the otoliths consisted generally of a mixture of aragonite and calcite. There is no explanation at hand for this general distribution of the calcium carbonate polymorphs. The body temperature alone cannot possibly be the main reason for the precipitation of one or the other form of carbonate.

A more detailed inspection of the results obtained from the different classes of gnathostomes reveals some interesting features. In elasmobranchs a unique situation is encountered. This is the only class in which the labyrinth is in contact with the outer environment through the endolymphatic duct. It is obvious that such a connection may influence the composition of the endolymph. However, the function of this passage *in vivo* is not established. We know that foreign particles, and thus also sea water, do enter the labyrinth of some species; but it is not known whether the endolymphatic duct of those species which possess only endogenous statoconia will also allow sea water to pass into the labyrinth. Clearly, differences in the local ionic environment in which the otoliths are formed and suspended may result in differences in their composition. It is worth mentioning that in those species of sharks and rays in which only endogenous statoconia were found, these were all very similar, consisting of minute aragonite bodies of a polycrystalline nature. In one species, additional statoconia, consisting of calcium carbonate monohydrate and calcite, were found. In the only elasmobranch here investigated, which certainly possessed an open ductus endolymphaticus, allowing sand to enter the labyrinth, the endogenous statoconia were completely different from those of the other elasmobranchs. On the whole, the elasmobranchs thus show a considerable heterogeneity in the crystallographic properties of their endogenous statoliths. It is anticipated that when more elasmobranch species are investigated, still other minerals may be encountered, owing to the role of the endolymphatic duct in ionic transport.

In the class of Actinopterygii, all species investigated belonging to the superorders Chondrostei and Holostei had statoconia composed of vaterite, a mineral which was not found in the labyrinth of any other group of vertebrates. Moreover, the statoconia always occurred together with large statoliths, another unique feature among gnathosotomes. In these respects, the Chondrostei and Holostei seem to form a perfectly homogeneous group. However, in this group, the sturgeons deviate from the other species in that their statoliths and microstatoliths consist of vaterite, while the statoliths of *Polypterus*, *Amia* and *Lepisosteus* are of the common teleost type, that is, of pure aragonite. It appears, therefore, that *Polypterus* is chemically closer related to *Amia* and *Lepisosteus* than to *Acipenser*. The rest of the actinopterygian fishes investigated, *i.e.*, all the teleosts, had statoliths of the same structure and composition, thereby forming a completely homogeneous group. It is commonly realized that the classification of actinopterygians is far from satisfactory. To take an example, the correct place of *Polypterus* in the system of fishes is much debated. In Figure 5, it has been placed all by itself in the group Cladistia, in accordance with the latest views on the evolution of the vertebrates (Jarvik, 1960).

In the class of Crossopterygii, which is of special interest since it is regarded as the origin of land vertebrates, the two orders Coelacanthini and Dipnoi had otoliths of entirely different types. Thus, in the Coelacanthini, represented by *Latimeria chalumnae*, the static body examined was a polycrystalline statolith of the same structure and composition as found in teleosts. The lung-fish, *Protopterus*, on the other hand, had statoconia consisting of perfect single crystals of aragonite. This type of static bodies is otherwise found only in tetrapods. Although most modern taxonomists place the Coelacanthini and the Dipnoi in the same class, some scientists are not satisfied with this classification. Certain authorities regard the Dipnoi as an entirely separate class (Berg, 1958; Jarvik, 1960). This view has been adhered to in Figure 5. That many similarities exist between Dipnoi and Amphibia has been pointed out by Säve-Söderbergh (1934); there are, however, several dissimilarities which make a closer relationship between these groups improbable. Because of the restricted information available concerning the nature of the otoliths of crossopterygians we are unable here to draw any conclusions as to the significance of the present findings. Since coelacanths similar to *Latimeria* existed 400 million years ago, it seems probable that the Coelacanthini had developed at an early stage the highly specialized static bodies found in teleosts. Such otoliths are not likely to be the forerunners of the static bodies of land vertebrates. Therefore, paleozoological information on the type and structure of the otoliths of the primitive Rhipidistian fishes (Porolepiformes and Osteolepiformes), which are regarded as closest to the possible tetrapod ancestors (Jarvik, 1960), is badly wanted. It should not be too difficult to judge whether statoliths or statoconia were present in this group of fishes, as it can be anticipated that at least the size and shape of the otoliths should be very well preserved.

In the class of Amphibia, only statoconia in the form of single crystals of aragonite were found. From this point of view the amphibians form a completely homogeneous group. The curious composite "statoliths" of the axolotl, a larval form of a salamander, encourage further studies. It would be of special interest to know whether the larvae of lung fishes possess similar otoliths.

The otoliths of Reptilia are interesting in that they seem to form an intermediate stage between amphibians on the one hand and birds and mammals on the other. In most species investigated, the statoconia consisted of a mixture of aragonite and calcite crystals. The highest percentage of calcite was found in the subclass Anapsida, which is supposed to be the living group closest related to mammals.

Finally, in the classes of birds and mammals, all species investigated possessed statoconia of the same nature, *viz.*, single crystals of calcite.

The correlation between the nature of the otoliths in different vertebrates and the commonly accepted classification is striking. Disagreements were found in a few instances only, and these were in fact restricted to groups where classification is still considered controversial. Even if the value of an investigation of this kind for the study of vertebrate phylogeny is limited, it is hoped that further studies, especially on paleozoological material, may aid in the classification of certain critical groups.

Apart from their possible phylogenetic significance, there are other interesting aspects of otoliths which certainly need further investigation. Why do vertebrates which have an abundance of calcium phosphate in their skeletons produce pure calcium carbonate in a single site in their body? From the functional point of view it would seem as if apatite, which has a higher density than any calcium carbonate, should be better suited for the function of the otolith organ. That apatite can be used instead of calcium carbonate is shown by the cyclostomes.

From studies on the domestic fowl it is known that the statoconia are deposited very early during development, even before the start of the calcification of the skeleton (Herzog, 1925). But it is not known if the statoconia, once formed, persist, or if there is a continuous production and perhaps also destruction of statoconia throughout the life span of an animal.

Also, it is completely unknown why some vertebrates have more statoconia in their labyrinths than others. The total weight of the statoconia in a small ray or frog is thus many thousand times that of the statoconia of a man or a whale. In a frog, which has the endolymphatic sacs packed with statoconia, all these small bodies cannot possibly be needed for the function of the otolith organ.

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SUMMARY

1. The crystallographic properties of otoliths from 58 vertebrate species were investigated by means of polarization microscopy and x-ray diffraction.

2. The otoliths occur as statoconia, microstatoliths, and statoliths. Usually only one kind is present in the labyrinth but in some vertebrates a combination of two or all three types may be found.

3. While statoliths and microstatoliths always are polycrystalline, statoconia may either be polycrystalline or single crystals.

4. Five different minerals, *viz.*, apatite, calcite, aragonite, vaterite and calcium carbonate monohydrate, compose the endogenous otoliths in the vertebrate labyrinth. Some elasmobranchs have in addition exogenous statoconia consisting of sea sand.

5. The distribution of statoliths and/or statoconia, their texture and their composition within the vertebrate series, show remarkable consistencies; within each class the same kind of static bodies is usually present.

6. Some crystallographic and biological aspects are discussed and it is suggested that the findings may be of some value for the study of the phylogeny of vertebrates and also may aid towards a better understanding of the function of the otolith organ.

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DIGESTIVE ENZYMES OF THE ECHIUROID, OCHETOSTOMA ERYTHROGRAMMON

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Apart from the observation of Gislén (1940) that the brown juice from the midgut of *Echiurus echiurus* contained a proteolytic enzyme, an amylase and an esterase, work on the enzymes of echiuroids is lacking.

In the present study observations were made on the digestive enzymes and their distribution along the gut of *Ochetostoma erythrogrammon.*

Materials and Methods

Specimens of *Ochetostoma erythrogrammon* from the intertidal muddy sand of the west coast of Singapore Island were starved in filtered sea water in the laboratory for at least four weeks, to clear the gut of faecal pellets and to allow the digestive fluid to accumulate.

The pH of the gut fluid was determined by mixing it with an equal quantity of indicator solution and matching the color formed against a series of standards made up of indicator solution and buffers of known pH. The indicators used were brom-thymol blue and thymol blue.

A high incubation temperature increases both the catalytic activity and the rate of inactivation of an enzyme (Dixon and Webb, 1958). Because of the small quantity of gut fluid and enzyme extracts, temperatures of 35° C. and 40° C. were employed to produce sufficient digestion products for titration within three hours. Presumably, these temperatures, being near to the maximum temperature experienced by *Ochetostoma* in nature, did not greatly inactivate the enzymes. At ebb tide on a windless sunny day, the shallow water of tide pools bathing these animals often reached a temperature of 37° C.

In the proteolytic enzyme experiments 0.1 ml. of gut fluid was incubated at 40° C. with a mixture of 1 ml. Clark and Lubs buffer solution and 1 ml. of 6% gelatine. The proteolytic activity was determined by titrating the carboxyl groups released with 0.01 N alcoholic potassium hydroxide solution and using 1% alcoholic thymolphthalein solution as indicator (Tauber, 1950). Control experiments at all pH values were carried out. Tests on egg albumen, casein, fibrin, nylon fibers and spongin fibers were made.

In the amylase experiments 0.15 ml. of gut fluid was incubated at 35° C. with a mixture of 0.5 ml. Sorensen's phosphate buffer and 1 ml. of 2% soluble starch solution. The amylolytic activity was determined by recording the time taken by the enzyme-substrate mixture to become colorless when tested with light-yellow solution of iodine in 2% potassium iodide solution.

For other carbohydrase experiments 0.15 ml. of gut fluid was incubated at 40° C. with 5 ml. of Sorensen's phosphate buffer at pH 7.0 with 20 ml. of 2%

inulin solution or 5% solutions of lactose, maltose or sucrose. The enzymic activity was determined by titration with Barfoed's solution or Benedict's solution. Phenylhydrazine tests were carried out for confirmation. Other tests included cellulose fibers (from filter paper) and glycogen as substrates.

Esterase activity at various pH values was determined by titrating with 0.01 N alcoholic potassium hydroxide digests containing midgut fluid or gut-wall extract and the substrate benzyl n-butyrate (Tauber, 1950). Olive oil and ethyl butyrate were also used as substrates in other esterase tests (Hawk, Oser and Summerson, 1947).

RESULTS

In this study the gut is subdivided anteriorly simply into the muscular pharynx, with radiating muscular strands, and the less muscular oesophagus, which swells up posteriorly into a crop. This is encircled by a loop of blood vessel and is succeeded by the foregut, which has throughout its length a ciliated groove. The midgut which follows has a siphon. The hindgut has a ciliated groove but no siphon and ends in a muscular bulbous cloaca.

Freshly collected specimens had accumulations of light-yellow to purplish fluid at various regions of the midgut. After starvation in filtered sea water for

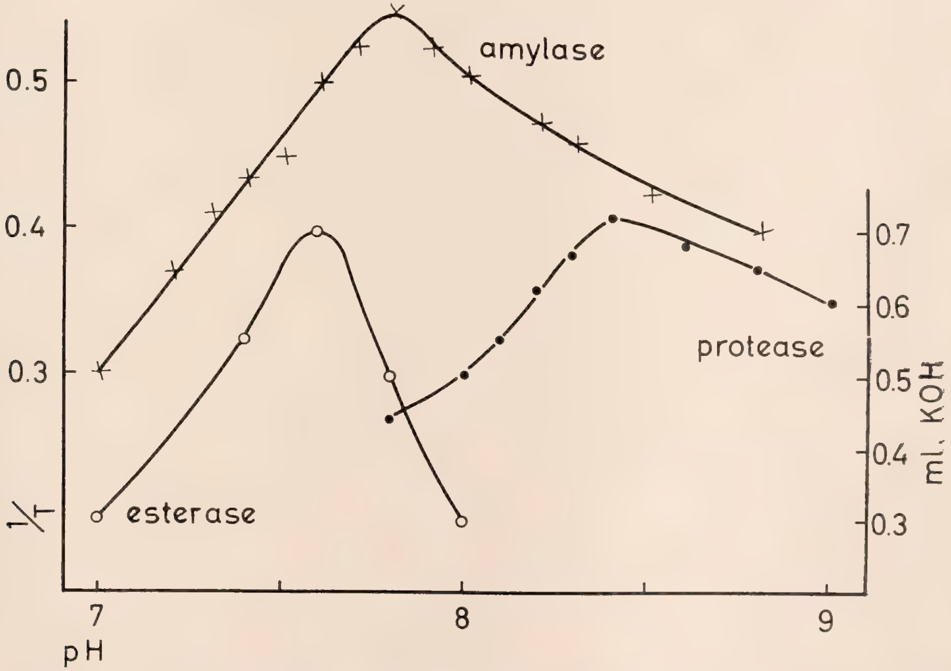

FIGURE 1. The pH activity curves of amylase, protease and esterase from the midgut fluid of *Ochetostoma erythrogrammon*. 1/T = reciprocal of time taken by the amylase-starch mixture to become colorless with iodine; ml. KOH = titer of 0.01 N KOH per 0.1 ml. digest of protease-gelatine and per ml. digest of esterase-benzyl n-butyrate.

several weeks the midgut fluid seemed to increase. Occasional accumulation of fluid also occurred in the adjoining parts of both the hindgut and the foregut.

By pooling the midgut fluids of 18 small, 16 medium, 10 large and 7 large starved specimens separately, averages of 0.09, 0.14, 0.23 and 0.33 ml. of fluid per worm, respectively, were obtained. The pH of midgut fluid from 10 starved specimens had a range of 7.0–8.2 with an average of 7.5, the lighter-colored fluid tending to be more alkaline.

The midgut fluid, extracts of the hindgut, midgut and foregut showed proteolytic activity, which decreased in that order per unit weight of gut tissue with gelatine as substrate. Extracts of pharynx, oesophagus and crop had no proteolytic activity. The pH activity curve of the proteolytic enzyme in the gut fluid shows a maximum at pH 8.4 (Fig. 1). Other proteins digested include casein, egg albumen and fibrin. Spongin and nylon fibers were not digested.

The midgut fluid showed amylolytic activity which reaches a maximum at pH 7.8 (Fig. 1). The starch solution undergoing this enzymic hydrolysis, when tested at intervals with iodine test solution, showed a change from blue through shades of mauve and pink to colorlessness, indicating a diminution in the size of the starch molecules to colorless achroodextrins and maltose. This was identified as maltosazone with the phenylhydrazine test. Glycogen was also digested. The amylolytic activity of 5% extracts of the midgut, foregut, crop and hindgut decreased in that order at pH 7.8. The extracts of the pharynx and oesophagus showed no amylolytic activity.

The midgut fluid, extract of midgut and extract of hindgut contained an esterase which hydrolyzes olive oil, benzyl n-butyrate and butyl acetate. In an individual specimen the esterase activity decreased in the following order: entire midgut fluid, extract of entire hindgut, extract of entire midgut. Extracts of the pharynx, oesophagus, crop and foregut showed no esterase activity (Table I). With benzyl n-butyrate as substrate the esterase activity reached a maximum at about pH 7.6 (Fig. 1).

TABLE I

Wet weight, and relative strength of digestive enzymes, of the various gut regions of Ochetostoma erythrogrammon

	Pharynx	Oesophagus and crop	Foregut	Midgut	Hindgut	Midgut fluid
Average wet weight of gut (minus gut contents) of 5 large specimens in mg.	0.7	1.3	4.8	41	53	
Protease	—*	—	+*	++	++	+++
Esterase	—	—	—	+	++	+++
Amylase	—	++	+++	++++	+	+++++
Maltase	—	—	—	—	+	+ or −

* — indicates absence; +, presence, with more crosses for greater strength.

Hindgut extract and some samples of midgut fluid contained a weak maltase. The midgut fluid had no inulase, cellulase, sucrase or lactase.

Discussion

Ochetostoma erythrogrammon is a detritus-feeder (Chuang, 1962). Its midgut fluid contains a protease, esterase and amylase as in *Echiurus echiurus* (Gislén, 1940). Brasil (1904), Nicol (1931) and Dales (1955) also found these in extracts of the gut wall of the polychaetes *Pectinaria koreni*, *Sabella pavonina* and *Amphitrite johnstoni*, respectively. The protease and amylase are strong in *O. erythrogrammon* and the three polychaetes mentioned above, while in *E. echiurus* both amylase and esterase are weak (Gislén, 1940).

The distribution and relative strength of the important enzymes along the alimentary canal of *O. erythrogrammon* are summarized in Table I. Protease is more extensively distributed than esterase. The most widespread enzyme is the amylase, which is absent only in the short length of the gut anterior to the foregut; its concentration is least in the hindgut, where maltase occurs. There is thus a localization of enzymes splitting higher and smaller products of starch in this animal as in vertebrates. In *Amphitrite johnstoni* the protease, amylase and esterase are confined to the fore-stomach and fore-intestine (Dales, 1955), while in *Pectinaria koreni* amylase occurs only in the most anterior part of the midgut, and protease, in the second part of the midgut (Brasil, 1904). Digestive enzymes are therefore more widely distributed along the alimentary canal of *O. erythrogrammon* than in the polychaetes.

Amylase and maltase mostly occur together in invertebrates (Vonk, 1937). This is so for the hindgut of *O. erythrogrammon* but not for the oesophagus, crop, foregut and midgut (Table I). Some samples of midgut fluid contain maltase, others not, indicating either the secretion of maltase in the midgut of some specimens or the escape of hindgut maltase into the midgut lumen, which continues uninterruptedly into the hindgut lumen without an intervening sphincter. The amylase in both *O. erythrogrammon* and the polychaete *S. pavonina* hydrolyzes both starch and glycogen as amylases from other sources do (Tauber, 1950).

The pH of the gut contents varies from one end of the alimentary canal to another. In *O. erythrogrammon* it rises from 7.6 in the crop to a maximum of about 8.2 in the midgut and then falls to 8.0 in the hindgut. In *S. pavonina* it rises from 6.8 in the first tenth to thirtieth segment to a maximum of between 8.0 and 8.4 at about the one hundredth segment, falls suddenly to 7.0 and then more slowly to 6.0 (Nicol, 1931). In *Amphitrite johnstoni*, however, it dropped from 7.0 in the oesophagus to about 6.0 in the fore-stomach, this minimum being maintained throughout the following region until the hind-intestine, from where it rises from 6.2 to a maximum of 7.2 at the rectal end (Dales, 1955). Dales also found that starved worms had a higher pH throughout the length of the gut. The higher values of the gut contents in *O. erythrogrammon* are associated with higher pH optima for the chief digestive enzymes. The pH optima of protease, amylase and esterase in *O. erythrogrammon* lie at 8.4, 7.8 and 7.6, respectively, against 8.0, 6.8 and 7.4 for *S. pavonina* (Nicol, 1931). The activity of the protease on gelatine in *O. erythrogrammon* decreases more slowly on the alkaline side of the pH range than on the acid side as in *S. pavonina* (Nicol, 1931). The esterase in both is active over a narrow pH range.

The specificity of the enzymes of *O. erythrogrammon* is similar to that of the polychaete *S. pavonina.* In both the amylase digests both starch and glycogen, the protease hydrolyzing gelatine, casein, fibrin and egg albumen, the esterase splitting neutral fat, such as olive oil, and esters, such as ethyl butyrate (Nicol, 1931).

The midgut and the hindgut in *O. erythrogrammon* constitute the greater part of the alimentary canal (Table I), as in *Echiurus echiurus* (Spengel, 1880), *Urechis chilensis* (Seitz, 1907) and *Urechis caupo* (Fisher and MacGinitie, 1928). They also contain many digestive enzymes. Fluid accumulates in the midgut as in *E. echiurus* (Greef, 1879), and occasionally also in the adjoining parts of the foregut and hindgut. The long coiled midgut, with its wide lumen often distended with a large amount of sand and fluid, is presumably the most important site of digestion in *O. erythrogrammon*, as Greef (1879) suggested for *E. echiurus.* The hindgut of *Ochetostoma*, with its multitude of enzymes, may also play an important role in digestion. The foregut may be less important and the more anterior regions of the gut presumably do not contribute much to actual digestion of food because of their short length and poor enzyme content.

The midgut fluid of *O. erythrogrammon* has a high concentration of digestive enzymes. For instance, 0.05 cc. of midgut fluid has an amylolytic activity of 30 mg. of midgut wall (wet weight) and gelatine-hydrolyzing power of 200 mg. of midgut wall. Similarly, the midgut fluid is richer in esterase than the midgut or hindgut wall. Seitz (1907) found secretory granules in the epithelial cells of the midgut in *U. chilensis.* Presumably, the midgut fluid of *Ochetostoma* represents an accumulation of continuous enzyme secretion over the long period of starvation, and is therefore more potent than extracts of the gut wall, which contain only what was in the cells at the time of extraction. Presumably, *O. erythrogrammon* digests its food mainly extracellularly in the midgut lumen. Largely on histological grounds, Dales (1955) also presumed extracellular digestion in *Amphitrite johnstoni.*

From the digestive enzymes of the midgut fluid, *O. erythrogrammon* appears able to digest food of both animal and plant origin from the detritus collecting on the wall of the burrow and on the surface mud outside (Chuang, 1962). However, the lack of such carbohydrases as cellulase, lactase, sucrase and inulase must considerably reduce its ability to utilize detritus of plant origin. Nicol (1931), finding no lactase, maltase, cellulase or sucrase in *S. pavonina*, also concluded that plant detritus was probably not utilized by *Sabella.*

SUMMARY

1. The digestive enzymes of *Ochetostoma erythrogrammon* were investigated. They include a strong amylase, a strong protease, a moderately strong esterase and a weak maltase. No cellulase, sucrase, lactase or inulase were found.

2. The midgut fluid accumulated during starvation in the lumen of the entire midgut. It proved a richer source of digestive enzymes than extracts of the various regions of the gut. Amylase occurred along the entire gut wall except

the pharynx. Protease occurred in the foregut, midgut and hindgut, esterase in the midgut and hindgut, and maltase only in the hindgut.

3. Both amylase and protease are active over a wide range of pH values, but the esterase has a more restricted range. The pH optima of these lie on the alkaline side of neutrality, with the protease having the highest pH optimum of 8.4 for the substrate gelatine.

4. The gut contents had an alkaline reaction, the pH rising from the crop to a maximum in the midgut and then dropping slightly in the hindgut. The pH of the midgut fluid in starved specimens varied between 7.0 and 8.2.

5. Digestion in *O. erythrogrammon* is presumably extracellular, occurring chiefly in the midgut. The ability of *Ochetostoma* to utilize plant detritus must be considerably reduced by its lack of carbohydrases other than amylase and a weak maltase.

6. The enzymes of *O. erythrogrammon* are discussed and compared with those of polychaetes.

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THE MECHANISM OF THE SHADOW REFLEX IN CIRRIPEDIA. I. ELECTRICAL ACTIVITY IN THE SUPRAESOPHAGEAL GANGLION AND OCELLAR NERVE[1]

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The protective withdrawal-closure reaction of barnacles in response to shading was first alluded to by Coldstream (1836), who noted that *Balanus* would close when the hand was passed over it at a distance of twelve or fourteen inches and wrote ". . . we could not but conclude that the animal was made sensible through the medium of the air, of the presence of some foreign body, and fearing danger, closed its shell for self-protection . . ." (p. 688). Von Siebold (1848, p. 343) correctly surmised that this reaction was caused by shading, but at the time denied the existence of eyes in adult cirripedes. Leidy (1848) discovered ocelli in *Balanus rugosus* (probably *B. crenatus*), and Darwin (1851, 1854) described these structures in other balanoids and in *Lepas anatifera.* Gruvel (1893) produced a rather thorough study of the morphology of the nervous system of *L. anatifera,* including considerable detail on the eye and associated ganglia, and Nussbaum (1890) noted the occurrence of a pair of ocelli in *Mitella* (= *Pollicipes*) *polymerus.*

Since Leidy's discovery of the ocelli in adult barnacles, it has been assumed that they were the sole photoreceptors and thus instrumental in the shadow reflex. The only attempt to establish the involvement of the ocelli in the reflex, however, is that of Fales (1928) who reported that the ablation of these structures in *Balanus eburneus* extinguished the reflex. Because of their internal location considerable injury must have occurred during removal, and the failure to respond may have had other causes.

The behavior of *Balanus* in response to shading under various conditions of illumination and other environmental factors was extensively reported by von Buddenbrock (1930), who pointed out that there is only a response to a sudden decrease in illumination, and that there is no overt short-term response to an increase in illumination.

The existence of a stereotyped behavioral response of the pure "off" type, coupled with what appeared to be a few millimeters of primary sensory neuron (Gwilliam, 1962) between photoreceptor and ganglion, suggested cirripedes as being good material in which to attempt an analysis of a behavioral response from primary receptor to the motor neurons activating the muscles involved. The

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recent report of Hoyle and Smythe (1963) on the giant muscle fibers of barnacles also suggests the possibility of intracellular recording of muscle potentials while the animal is being subjected to sudden changes in illumination.

This paper reports the results of a survey of four species of barnacles and represents the beginning of what is hoped will eventually be an analysis of the various factors involved in the shadow reflex from a neurophysiological point of view. A preliminary account of some parts of this work has been reported (Gwilliam, 1962).

MATERIALS AND METHODS

The following species of barnacles were used in the study: *Mitella* (= *Pollicipes*) *polymerus* (Sowerby), *Lepas anatifera* L., *Balanus cariosus* (Pallas), and *Balanus eburneus* Gould. *Mitella* and *B. cariosus* were collected as required from the central Oregon coast. *Lepas anatifera* and *B. eburneus* were provided by the Supply Department of the Marine Biological Laboratory, Woods Hole, Mass.

Details of the connections between photoreceptors and the supraesophageal ganglion were determined by dissection and methylene blue staining after it was ascertained physiologically that the structure in question was indeed the photoreceptor.

Electrical activity was recorded with platinum or platinum-iridium hook electrodes or with forceps electrodes. Signals were amplified with a conventional A. C. preamplifier and displayed on one beam of a Tektronix Type 502 dual beam oscilloscope. The second beam of the oscilloscope displayed the signal from a selenium photocell that was placed close to the preparation. Permanent records were made on a moving film with a Grass C-4 kymograph camera.

Shadows were cast with a sector disc driven by an electric kymograph motor, with a 35-mm. camera focal plane shutter, and manually. The light source used was a 6-volt tungsten lamp which was not controlled for absolute intensity, and the distance from the preparation was held constant only during a given experiment. Neutral density filters were used to vary intensity, as well as being used to cast shadows.

For medium bathing the preparation consisted either of sea water or *Balanus* Ringer (Hoyle and Smyth, 1963). Under these conditions and at room temperatures of 18–23° C. preparations were consistently viable for up to 12 hours without special precautions being employed. However, no single preparation was used for more than about three hours.

RESULTS

Structure

Figure 1 illustrates the relationships of the various photoreceptors to the supraesophageal ganglion in the species examined. The histological details of the photoreceptors themselves differ from species to species and will be reported in a separate communication. Some histological details of the eye of *Lepas anatifera* have been reported by Gruvel (1893), and of *Balanus eburneus* by Fales (1928).

As can be seen in the diagrams, the photoreceptor lies in the midline in all the species examined except *Balanus eburneus*. In this species the pair of ocelli

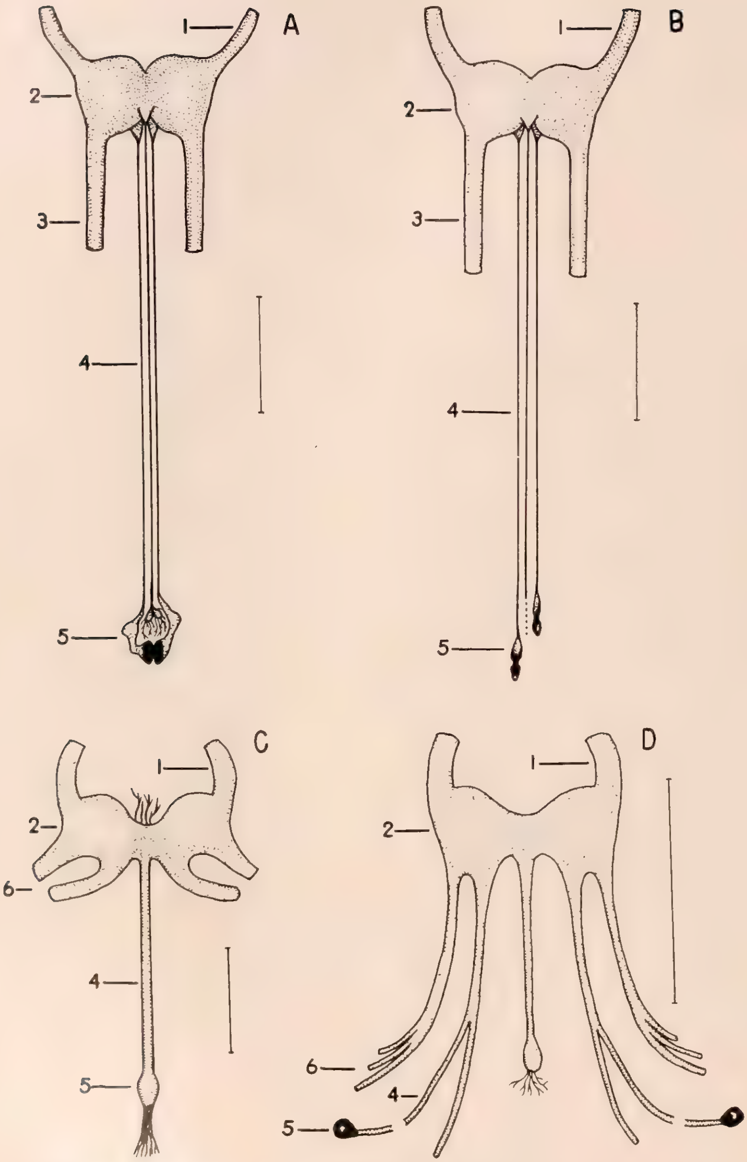

FIGURE 1. Diagrams of the relationship of the ocellus and the supraesophageal ganglion in the four species of barnacles studied. A, *Lepas;* B, *Mitella;* C, *Balanus cariosus;* D, *B. eburneus.* (1, circumesophageal connective; 2, supraesophageal ganglion; 3, stalk nerve; 4, ocellar nerve; 5, photoreceptor; 6, mantle nerves). Gap in ocellar nerve in D indicates the omission of 2–3 mm. of nerve. Scale markers = 1 mm.

are to be found in the mantle lateral to the body, just proximal to the edge of scutes. They are thus located in the inner lining of the shell "looking inward."

Electrical activity

(a) *Mitella polymerus.* Recordings from the stalk nerves of this species in light or after 15 minutes in the dark show a decidedly rhythmical pattern of bursts of activity as illustrated in Figure 2, A. Bursts often occur more frequently than those illustrated, but the general nature of the rhythmical activity is always similar. If a shadow is cast on the preparation in light just after one of these spontaneous bursts, there is a more prolonged response, followed by a longer than expected period of silence, and no activity at the cessation of shading (Fig. 2, B). Multiple shadows of 300 milliseconds duration at two-second intervals elicited a response at each shadow, but adapted somewhat with succeeding stimuli although a definite response was still evident after 30 such shadows (Fig. 2, C). Two hundred millisecond shadows at 2.5-second intervals failed to give repetitive responses, and 100 millisecond shadows at one-second intervals gave a significantly smaller initial response and failed to summate. Shadows of shorter duration failed to elicit any response. The relationship between shadow duration threshold and light intensity was not investigated in this species.

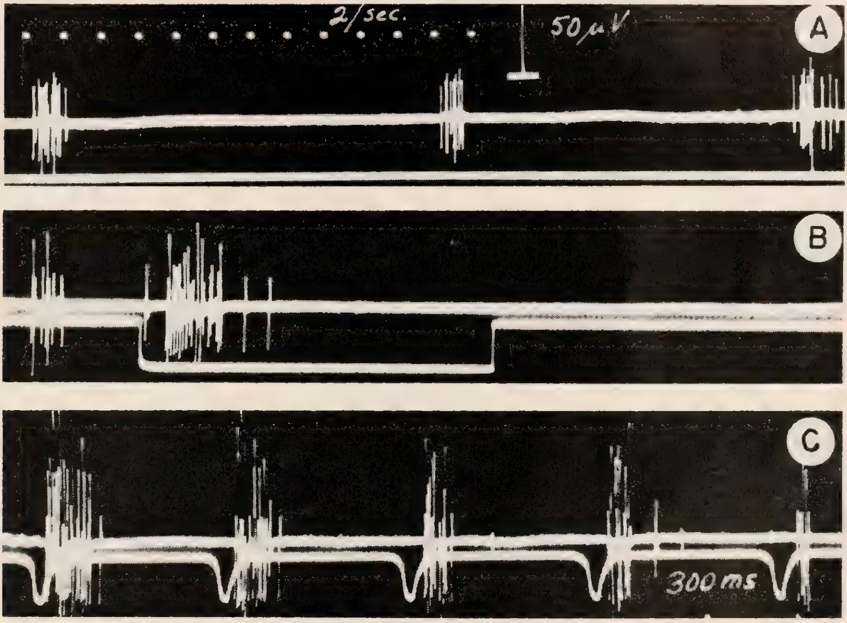

FIGURE 2. *Mitella.* Electrical activity recorded from the stalk nerve. A, Spontaneous activity in the dark. B, The "off" response. C, The effect of multiple shadows of 300-millisecond duration. In this record a response was still evident after 30 such shadows. Downward deflection of lower trace indicates "off," upward deflection "on" in this and all records to follow.

The fact that shadow "intensity" and magnitude of the response are related is illustrated in Figure 3. Shadows were cast with gelatin neutral density filters having per cent transmission characteristics indicated in the upper right hand corner of each record. It can be seen that cutting out 60% of the incident light results in what is essentially a full shadow reflex (compare Figure 3, C and F).

(b) *Lepas anatifera.* This species exhibited responses similar to those of *Mitella polymerus*, but it was also possible to record directly off the ocellar nerve in *Lepas* (Fig. 4). Records from the circumesophageal connectives and stalk nerves showed considerable spontaneous activity, but there was none of the regular rhythmic activity of the type shown by *Mitella* stalk nerves. This may reflect the rather different behavior patterns of the two animals. *Mitella* is a barnacle that lives attached to rocks, along with *Mytilus californianus.* It feeds by extending the cirri into a moving stream of water during high tide and is exposed at low water, during which time the cirri are inactive. Even during feeding, *Mitella* does not exhibit the regular extension and retraction "fishing" activities so typical of sessile barnacles, and it is possible that the regular rhythmical activity observed in the stalk and mantle motor nerves serves to provide regular pulsations of the muscles that aid in blood circulation in these heartless crustaceans. *Lepas,* on the other hand, lives attached to ships and floating timber and is at all times submerged. *Lepas* feeds much as does a sessile barnacle, by the regular extension and retraction of the cirri, and this activity could serve as a blood pump.

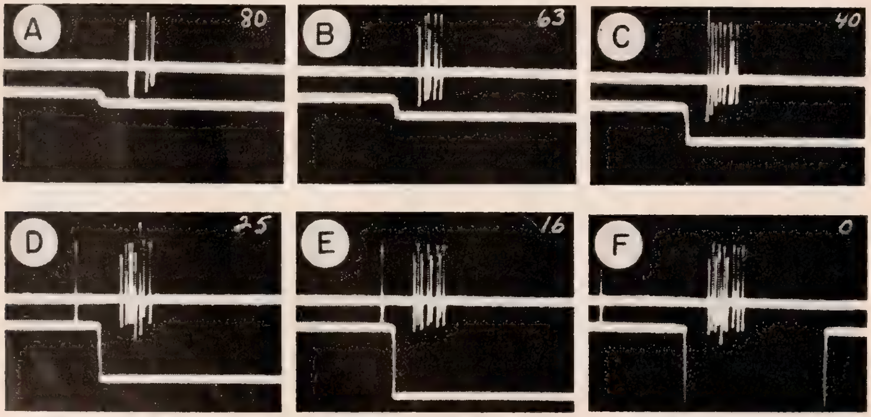

FIGURE 3. *Mitella.* The effect of casting shadows with neutral density filters. Figures in upper right hand corner of each record indicate per cent transmission of the filter used to cast the shadow. Recorded from the stalk nerve.

A shadow cast on a preparation such as that illustrated by Figure 4 elicits a burst of large-fiber activity in the circumesophageal connective (Fig. 4, A) and in the stalk nerves (Fig. 4, B, lower trace). A small positive deflection is recorded in the ocellar nerves proper at "off," and at "on" a much larger slow response, referred to as the electroretinogram (ERG), can be recorded (Fig. 4, C) which is graded and decremental. On no occasion have I been able to record spikes from the ocellar nerve.

Early in the investigation spikes were frequently recorded in the *bundle* bearing the ocellar nerves, but careful observation and dissection revealed three trunks in the bundle, only two of which led from the ocelli (Fig. 4, diagram). By recording from these trunks one at a time, it was established that the middle bundle gave rise to the spikes while the ocellar nerves gave only the ERG. If this middle nerve was severed at the level of the supraesophageal ganglion, the activity was arhythmical and unaffected by light or shadows. If recordings were made on the intact nerve, a "shadow reflex" was evident.

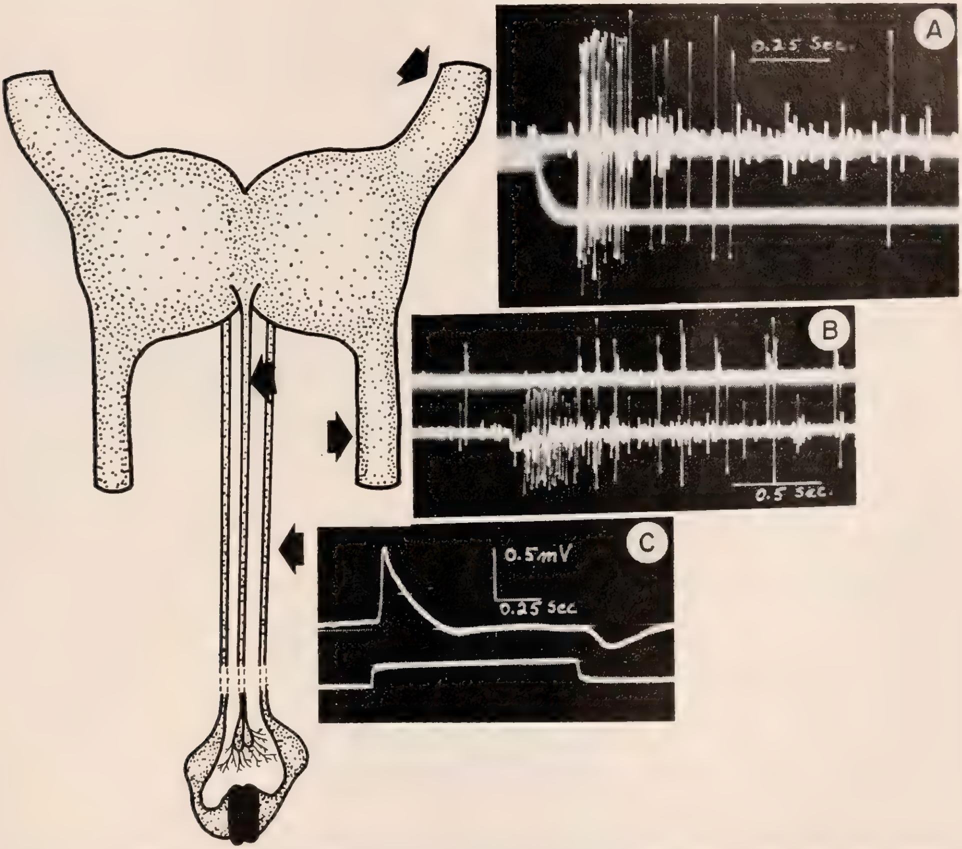

FIGURE 4. *Lepas.* Electrical events recorded from various positions in the nervous system. Arrows indicate recording sites. See text for explanation.

While the above seemed to rule out any possibility of this median nerve being responsible for the activity observed in stalk nerves and circumesophageal connectives, a further experiment was performed to establish the point. Leaving the median nerve intact, one could record from it and the stalk nerves simultaneously. A shadow was cast, and the resulting record demonstrates that the activity in the stalk nerve following shading (Fig. 4, B, lower trace; downward deflection of baseline signals "off") could not have been initiated by the activity in the median nerve (Fig. 4, B, upper trace) because it precedes it in time. Further, severing only the ocular nerves while leaving the median nerve intact abolishes the activity illustrated in Figure 4.

(c) *Balanus cariosus.* The photoreceptor in this large sessile barnacle consists of a ganglion lying close to the adductor scutorum muscle and connected to the supraesophageal ganglion (Figs. 1 and 5). It lacks the obvious ocellar pigments seen in other barnacles and differs very little in evident color from the rest of the nervous system in that region. Its function as the photoreceptor was established experimentally, and no other structure serving this function has been located in this species.

Considerable time was spent searching for an obvious ocellus, but none was found. The animal has a perfectly good shadow reflex, however, so that it was evident that a photoreceptor was present. To locate the structure, a circum-

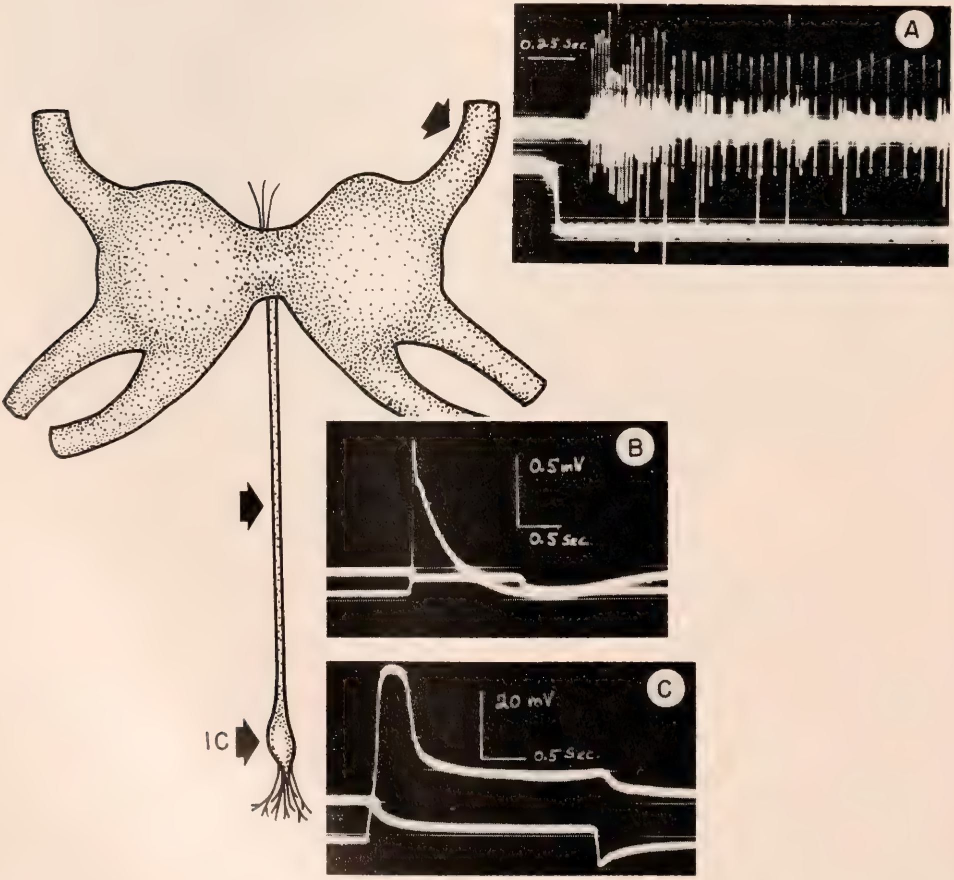

FIGURE 5. *Balanus cariosus.* Electrical activity recorded from the sites indicated by arrows. IC indicates record C is a presumed intracellular response. A and B, A. C. recording; C, D. C. recording.

esophageal connective was placed on a recording electrode, and the preparation was tested for the "off" response, then small areas were shaded, the general area of sensitivity quickly located, and the small ganglion was seen. Then, using a needle point to create a small shadow, it was demonstrated that shading the ganglion and only the ganglion gave the response. Crushing the ganglion or severing the tract to the supraesophageal ganglion obliterated the response in the circumesophageal connectives.

Under the recording conditions there was considerable small-spike spontaneous activity recorded from the circumesophageal connectives in *B. cariosus*. When a shadow was cast on the preparation there was a burst of large-fiber activity that persisted for a considerable period (Fig. 5, A) and ceased abruptly at "on." If left in the dark, the "shadow reflex" burst persisted, gradually diminishing in frequency for four to ten seconds.

Recordings from the ocellar nerve freed of other fibers always resulted only in an ERG similar to that recorded from the ocellar nerve of *Lepas* (Fig. 5, B).

At this time several attempts were made to penetrate cells in the photoreceptor ganglion with glass microelectrodes, and on a single occasion this appeared to be successful. One of the records obtained is reproduced as Figure 5, C. An attempt was made to record under varying light intensities, but I was unable to hold the cell for a sufficient length of time. The absence of spikes in this record supports the idea that these cells do not generate propagated action potentials, but under the circumstances this cannot be taken very seriously. Failure to hold the cell may indicate injury, and a failure to repeat the penetration has made it impossible to gain further information. The absence of spikes in such a record may indicate injury or that spike generation takes place in the axon and does not invade the soma. The electrode was tested with light flashes before and after the record was made to be certain the potential was not due to the action of light on the electrode itself.

Figure 6 illustrates the relation between ERG amplitude (recorded from the severed ocellar nerve) and light intensity. It further emphasizes the fact that the primary event is an "on" response, while the behavioral event takes place only at "off." This suggests that the activity of the photoreceptor transmitted via the ocellar nerve inhibits cells in the supraesophageal ganglion, and this inhibition is released at "off."

Failure to record spikes from the ocellar nerve was originally viewed simply as a technical failure in recording. However, after numerous attempts at recording under various conditions (in air, in oil, with partially dried preparations to minimize shunting, in carefully subdivided bundles), I was forced to consider the possibility that propagated spikes did not occur in the ocellar nerve and that the information was transmitted via electronic spread. Histological examination of the photoreceptor ganglion and the axons leading to the supraesophageal ganglion indicates that there are about eight cells present in the ganglion, and no synapses occur until the supraesophageal ganglion, which may be as distant as 4 mm. in a large individual. This is a very long distance for the phenomenon of electrotonic spread to be effective, but the following experiment suggests that this is indeed the case.

In *Balanus cariosus* it is possible to make a preparation in which the supra-

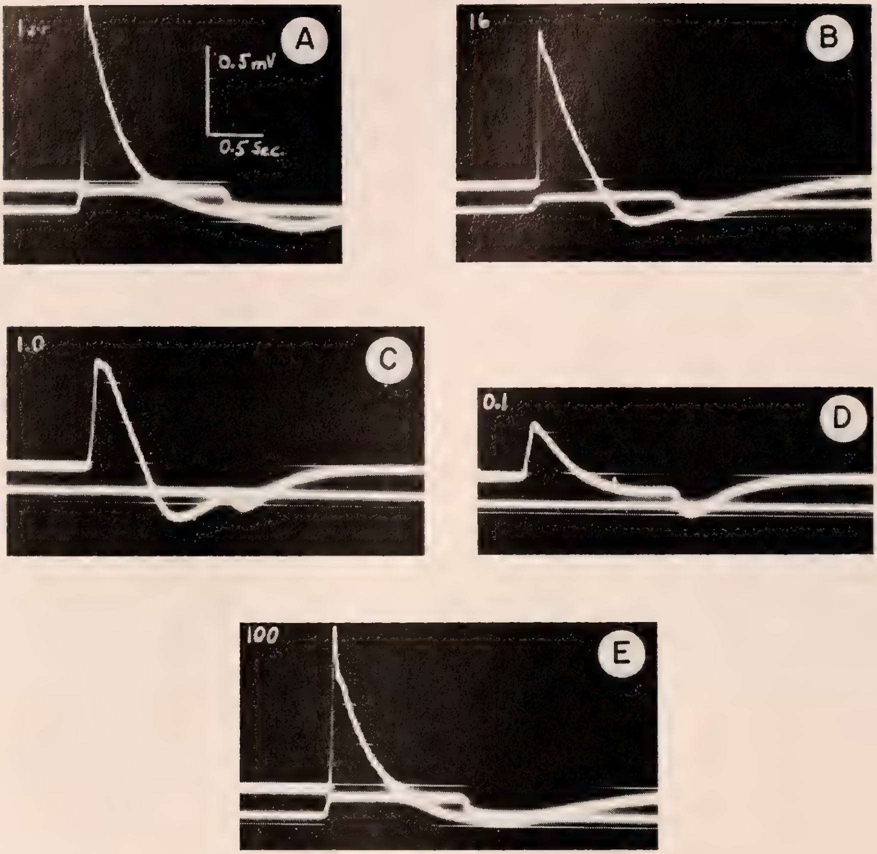

FIGURE 6. *Balanus cariosus.* The effect of light intensity on the externally recorded ERG. Recorded from the severed ocellar nerve. Per cent transmission of light flash indicated in upper left hand corner of each record. Photocell monitor failed to record in C and D. A. C. recording.

esophageal ganglion is cut free from the body of the barnacle, leaving it attached only by the ocellar nerve. The supraesophageal ganglion can then be lifted free of the bathing medium while the photoreceptor ganglion is still immersed, and recording can be accomplished from the circumesophageal connective. The supraesophageal ganglion is kept viable either by lifting into oil or preventing it from drying by the application of bathing medium at intervals.

In this way it was possible to soak only the photoreceptor ganglion and its axons in a solution of procaine, and test periodically for the shadow reflex in the circumesophageal connectives. Then the supraesophageal ganglion could be lowered into the procaine and testing continued. The results of such an experiment are shown in Figure 7. Soaking the photoreceptor ganglion in 0.5% procaine for as much as 20 minutes (Fig. 7, A, B) did not abolish the shadow reflex, but within two minutes after lowering the supraesophageal ganglion into the procaine, spiking was virtually abolished (Fig. 7, C). The effect was reversible, for 30 minutes after washing, a shadow reflex was again obtainable (Figure 7, D).

This experiment establishes that procaine does prevent spiking in barnacle nerves, and other studies (*e.g.*, Katz, 1950) indicate it does not prevent generator potentials. The experiment does not prove that electrotonic spread is the mechanism for transmission, for there is no direct evidence that the procaine penetrates the photoreceptor ganglion. There is little reason, on the other hand, to assume procaine does not penetrate, for the receptor ganglion sheath is, if anything, less well developed than that around the supraesophageal ganglion. Cut ends of nerves leading to the supraesophageal ganglion may speed penetration in that region, so the experiment can only be regarded as supporting, but not proving, the hypothesis of electrotonic spread.

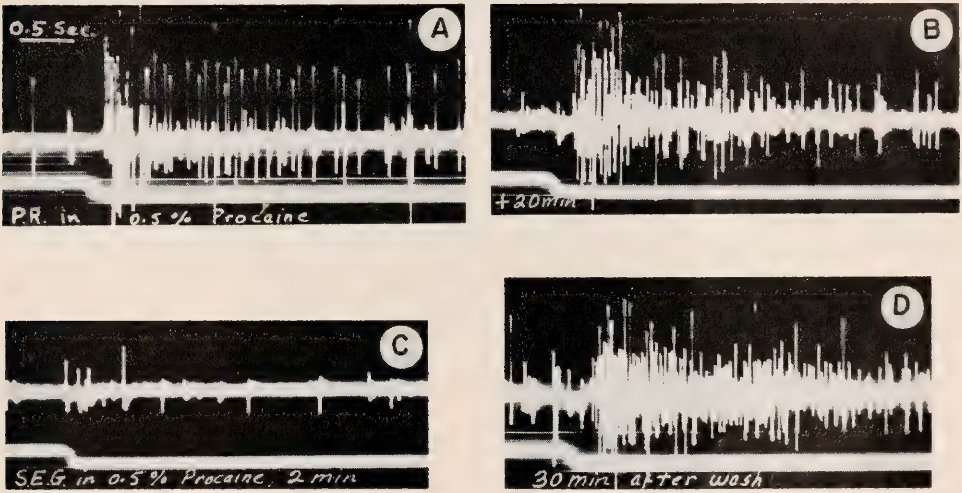

FIGURE 7. *Balanus cariosus.* Results of the procaine experiment described in the text. PR, photoreceptor; S.E.G., supraesophageal ganglion.

(d) *Balanus eburneus.* These are the smallest of the barnacles on which recording was attempted, but the laterally located photoreceptors were sufficiently interesting to make the attempt worthwhile. The general structure of the ocellus of *B. eburneus* is reported by Fales (1928), who illustrates the two sensory cells in each. These cells attain a size of 100 microns and appear to send their axons directly to the supraesophageal ganglion without synapses. There is also a median ganglion present as in *B. cariosus* which has photoreceptor activity.

Figure 8, A, B illustrates the activity recorded from the circumesophageal connective when a shadow is cast, and its cessation when the preparation is exposed to light. If the preparation is kept in the dark, the activity continues for about 40 seconds, decreasing exponentially in frequency from ca. 80/sec. in the first second to ca. 3/sec. in the last second. If multiple shadows are cast, bursts will continue to occur for more than 30 shadows. If, however, activity is recorded in the adductor motor nerve which originates in the ventral ganglion, there is a failure after about four similar shadows. This is in contrast to *Lepas,* where adaptation to multiple shadows appears to take place at the level of the supraesophageal ganglion rather than the ventral ganglion (Gwilliam, 1962).

Records taken from close to the ocellus result in an ERG similar in form to the others already noted, and a similar response is recorded from the median nerve (Fig. 8, C, D).

The fact that the median nerve in this species serves as a photoreceptor was discovered by accident. During a routine experiment to establish the obvious lateral ocellus as the sole photoreceptors, recordings were taken from the circumesophageal nerve before and after severing the ocellar nerve at the level of the ocelli, and a good shadow response was evident (Fig. 9, A). Then, all four lateral nerves were severed at the ganglion (the circumesophageal connectives having been severed previously), and the median nerve was cut *distal* to the small ganglion. Under these circumstances, a good shadow reflex was still obtainable (Fig. 9, B, C). The median nerve was then severed just proximal to the small distal ganglion, leaving a length of nerve which apparently itself was photosensitive, for a good response was still observed (Fig. 9, D). Finally, cutting the median nerve at the level of the supraesophageal ganglion abolished the response (Fig. 9, E).

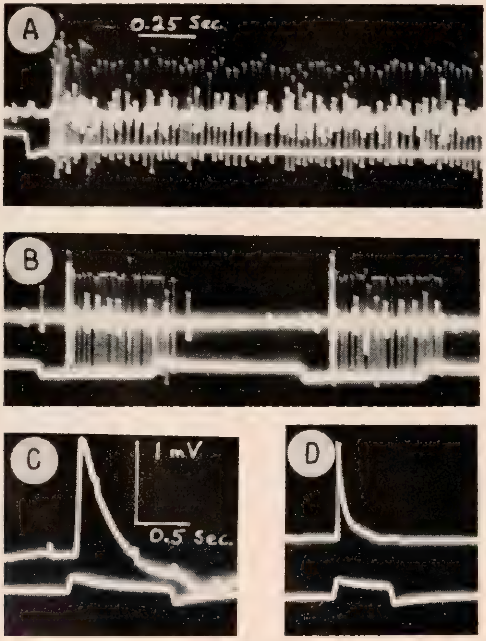

FIGURE 8. *Balanus eburneus.* Electrical events recorded from the circumesophageal connective (A and B), the ocellar nerve (C), and the "median" nerve (D). Time marker in A applies also to B; time and amplitude in C applies also to D. A. C. recording.

The function of this small median ganglion as a photoreceptor is not universal in *B. eburneus,* for not all preparations show this activity. It is even less common

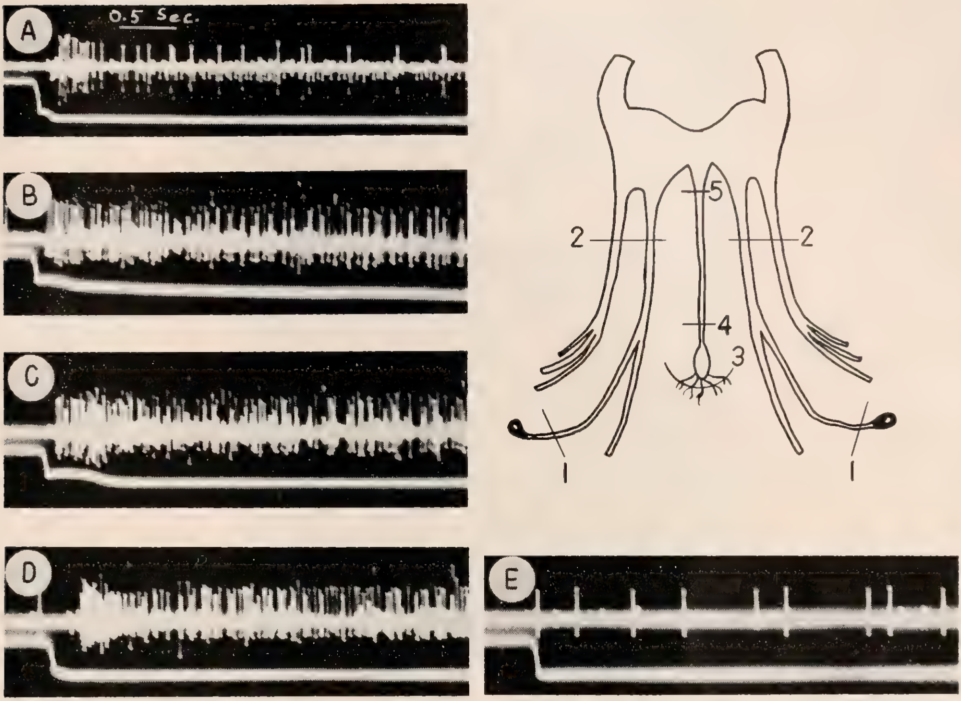

FIGURE 9. *Balanus eburneus.* The median distal ganglion and nerve functioning as a photoreceptor. Numbers on the diagram indicate the order of severing nerve tracts. A was recorded following cut 1, B following cut 2, etc. Circumesophageal recording.

for the median nerve itself to function in this way, but it is a factor which must be considered in ocellar ablation experiments.

If recordings are made from the entire severed bundle containing the ocellar nerve at the level of the supraesophageal ganglion (*i.e.*, preganglionic but with all distal structures including the ocellus intact), large spikes are often observed that frequently commence firing at "on" (Fig. 10, A). Such spikes have never been recorded close to the ocellus where only the ocellar nerves are present, but it is conceivable that the spiking locus is at some distance from the sensory cells.

While these potentials have not been completely ruled out as being activity from the photoreceptors, the following observations suggest that this is due to the activity of some other sensory system, possibly a mechanoreceptor.

(a) The activity occurs spontaneously at times in the absence of light (Fig. 10, B).
(b) The activity does not occur at "on" if all other input to the supraesophageal ganglion is interrupted.
(c) The activity frequently does not cease at "off" as would be necessary for the second order "off" response to occur (Fig. 10, C).
(d) Cutting the ocellar nerve at the ocellus does not abolish the spikes.
(e) Immersing the preganglionic fibers in procaine does not abolish the "off" response in the circumesophageal connectives.

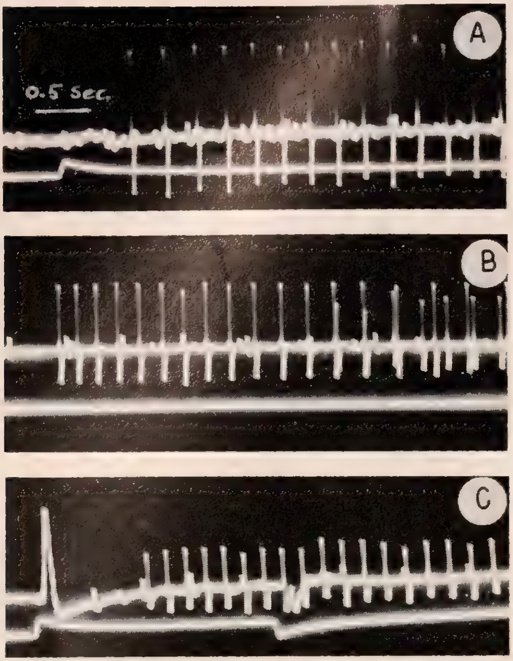

FIGURE 10. *Balanus eburneus.* Large-fiber activity from nerve tract containing the ocellar nerve recorded preganglionically. A, The "on" response. Note long latency. B, Spontaneous activity in the dark. C, Another "on" response with long latency and exhibiting the failure to stop at "off." Note elements of the ERG at "on" and "off."

It seems probable, therefore, that this fiber is involved in some other yet unknown sensory system.

DISCUSSION

Despite differences in detail of photoreceptor position and structure, all of the barnacles described in this study have essentially similar reactions at comparable points in the nervous system. This result is to be expected on the basis of the simple behavioral response common to all. As a result of this similarity, it is possible to generalize the discussion, pointing out differences where they occur and seem significant.

Beginning with the sensory output, all the evidence points to an "on" response, as would be expected from a photoreceptor, but this response takes the form of a simple electroretinogram consisting of a relatively large negative-going slow potential at "on" (from an active lead on the ocellar nerve with reference to an indifferent electrode in the medium) and a small positive deflection at "off." These ERGs were A. C.-recorded, and the form under these conditions may be interpreted as an initial large depolarization which quickly drops to a lower level that is maintained in the light. Upon cessation of illumination, the potential returns to the resting level, giving a small positive wave when A. C. recording is used.

An electroretinogram of this form is essentially the same as those recorded from insect retinular cells (Naka, 1961), *Limulus* retinular cells (Fuortes, 1958), and corresponds to the interpretation of the insect dorsal ocellus ERG given by Ruck (1961) without the complicating factors of the synaptic layer and post-synaptic ocellar nerve, *i.e.*, a transient "on" response and a sustained depolarization in light. The single presumed intracellular record from *B. cariosus* supports this interpretation, but under the circumstances it cannot be regarded as decisive.

The evidence also indicates that this ERG is the only form of photoreceptor activity transmitted to second order neurons in the supraesophageal ganglion and acts in an inhibitory fashion in these cells. Such a system would correspond to that described by Ruck (1962) as (p. 632) ". . . The general, perhaps evolutionarily primitive, synaptic relationship between retinula cells and the second order neurons. . . ."

The recent report of Naka and Eguchi (1962), however, will not permit the generalization that retinula cells do not generate spike potentials, for these authors have demonstrated that spikes do occur in retinula cells in the lateral eye of the drone honeybee. As yet there are no published intracellular records from the insect ocellus or indeed from the ocellus of any arthropod, and it is possible that in the more simply organized of these structures, the generator potential is the only form of activity. The evidence presented here suggests that this is the case in the very simple photoreceptor of barnacles. The single intracellular recording obtained matches closely in form those obtained from the worker honeybee (Naka and Eguchi, 1962) and the same explanations these authors offer for failure to record spikes may hold. However, the failure to record spikes in the ocellar nerve and the failure of procaine to obliterate the circumesophageal "off" response lends support to the idea that the barnacle photoreceptor cells do not generate all-or-nothing action potentials.

It should be noted that the barnacle ERG indicates a depolarization at "on," not the reverse as shown by Parry (1947) for the ocellar nerve of *Locusta*, even though both organisms show an "off" response in the circumesophageal commisures. Hoyle (1955) later demonstrated action potentials in the ocellar nerve of *Locusta*, and Waterman (1953) in the median ocellar nerve in *Limulus*. However, the insect ocellar nerve is composed of second order neurons, and the "off" response may be explained just as is the "off" response in the circumesophageal connectives in the barnacle. In the *Limulus* ocellus the origin of the spike potentials is obscure (Waterman, 1953).

The barnacle photoreceptor is presumably derived from the median naupliar eye, not the compound eyes of the cypris (Fales, 1928; Doochin, 1951). As such, its original position in the larva, lying physically very close to the supraesophageal ganglion, would render a mechanism of electrotonic spread quite plausible, just as in the insect dorsal ocellus where the first synapse is very close. The development of the adult eye might then be envisaged as involving an elongation of the photoreceptor cell axon as the ocellus assumed the new position, but retaining the "primitive" mechanism.

If the functional interpretation presented here is correct, and if there are indeed no synapses between the photoreceptors and the supraesophageal ganglion, then the barnacle "eye" is perhaps the simplest readily accessible photoreceptor yet known. The primary cells are few (two in each ocellus of *B. eburneus*, six to nine in the photoreceptor ganglion of *B. cariosus*, two in each ocellar ganglion of *Lepas*) and of relatively large size, and thus they offer an opportunity to study the primary receptors in isolation. We are currently engaged in attempts to record intracellularly from the large cells in the ocellus of *Balanus eburneus*.

Next, examining the output of the supraesophageal ganglion as recorded in the circumesophageal connectives in the two species of *Balanus,* we find that relatively few large cells respond at "off" in a tonic fashion, and this response does not adapt with multiple shadows. In *Lepas*, on the other hand, adaptation occurs relatively rapidly at this level (Gwilliam, 1962), as can be ascertained from recordings of the output at both the circumesophageal connectives and the stalk nerves. This may be a consequence of the fact that the stalk nerves originate in the supraesophageal ganglion and probably represents at best the third neuron in the chain, while in *Balanus* the nerve serving the adductor muscle originates in the ventral ganglion. When the motor output from the ventral ganglion to the adductor scutorum muscle in *B. eburneus* is examined, a rapid failure to respond to multiple shadows is seen. The difference in the recordings from the circumesophageal connectives in the two types of barnacles is not so readily explained but may have to do with the detailed distribution of motor cells. However, the fact that adaptation occurs just prior to the motor output stage would permit the barnacle to ignore repeated inconsequential shadows that must occur constantly while the animal is feeding in nature, but still be receiving information in the central nervous system about the changing light level in the environment.

In *Mitella* the failure of the motor output to the stalk nerves to adapt to multiple shadows is puzzling in the light of the rapid adaptation of the motor output of the other barnacles. The situation illustrated in Figure 2, C, however, is not always the case, for at different shadow durations and frequencies of shadow-casting adaptation does occur. It is possible that in this particular instance the spontaneous rhythm has been reset.

SUMMARY

1. The electrical activity of various parts of the nervous system under changing conditions of illumination in four species of barnacles has been studied.

2. The primary event at the photoreceptor is an "on" response that takes the form of a simple ERG similar to that described for the retinula cells in a number of other arthropods.

3. Evidence is presented that suggests that this information is transmitted to the second order neurons in the supraesophageal ganglion via electrotonic spread, but this cannot as yet be regarded as conclusive.

4. The second order neurons respond in a pure "off" fashion and are inhibited at "on." In *Balanus* this response, monitored at the circumesophageal connectives, is tonic, while in *Lepas* and *Mitella*, recordings at the same position display a phasic response. It is suggested that, at best, third order neurons are responsible for the phasic response in the latter two genera.

5. Evidence is presented that both the primary "on" response and the second order "off" response are graded with intensity of illumination.

6. Failure to respond to multiple shadows is evident in the motor output of all species except *Mitella*, in which the stalk nerves respond repeatedly to multiple

shadows. A possible explanation for the latter and a possible advantage of the former is offered.

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ACTIVATION OF RESPIRATION IN SEA URCHIN SPERMATOZOA BY EGG WATER[1]

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The fertilization of sea urchin eggs in sea water is preceded by interactions between the free-swimming spermatozoa and material that diffuses from the egg. The analysis of these interactions is of interest because of the likelihood that at least some of them are helpful or essential to fertilization. Attention has focused both on the soluble material of the egg, and on its various effects on spermatozoa (Metz, 1957; Wiese, 1961, for reviews); and in the present paper further observations of sea urchin spermatozoa in the presence of egg material are reported. Sea water containing material that has diffused from eggs is called "egg water," and in sea urchins, the best known component of egg water derives from the jelly that normally surrounds the egg. This "jelly-coat" is composed of a glycoprotein called fertilizin, which in solution causes the agglutination of sea urchin spermatozoa (Lillie, 1913; Tyler, 1956). Agglutination is thought to result from the formation of bonds between antifertilizin on the surface of spermatozoa, and fertilizin, each molecule of which is presumed to combine with two spermatozoa. The agglutination, however, is temporary or reversible, probably because of the swift degradation of fertilizin into a non-agglutinating or univalent form (Tyler, 1948; Hathaway and Metz, 1961).

Other reactions of sperm to egg water sometimes include (1) an enhancement of motility (Lillie, 1913, 1919), (2) changes in the respiratory rate (*e.g.*, Gray 1928b), (3) an acrosome reaction (Dan, 1952, 1956), and (4) a displacement of the mid-piece (Popa, 1927; Dan, 1954). The question arises whether these various reactions of spermatozoa can be attributed to fertilizin, or whether egg water contains other substances that affect the behavior and structure of spermatozoa. In some instances it is fertilizin, or a closely associated substance, that causes an increase in sperm motility. Fertilizin of *Strongylocentrotus purpuratus*, which had been precipitated and dialyzed, retained the property of activating motility (Tyler, 1955). In *Arbacia punctulata*, however, the activator of sperm motility differed from fertilizin by its volatility (Clowes and Bachman, 1921), and its diffusibility upon dialysis (Cornman, 1941). In *Echinocardium cordatum* the activator was also diffusible (Vasseur and Hagström, 1946). The activator in eggs of *Arbacia pustulosa* was reported to be the pigment echinochrome (Hartmann *et al.*, 1940), although this was questioned by Bielig and Dohrn (1950).

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[1] Contribution No. 185 from the Oceanographic Institute, Florida State University, Tallahassee. This work is from a dissertation submitted to the faculty of the Florida State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Purified echinochrome did not activate sperm motility in *S. purpuratus* (Tyler, 1939), or in *A. punctulata* (Cornman, 1941). An extract of egg water from *Psammechinus miliaris* caused an increase in motility and respiration of spermatozoa, and the substance affecting motility was tentatively identified as vanillin (Lybing and Hagström, 1957).

On the whole, little is known of agents in egg water that affect the respiration of spermatozoa. Oxygen uptake of spermatozoa increases after the addition of egg water in *Echinus esculentus* (Gray, 1928b; Carter, 1931; Vasseur, 1949), and *Strongylocentrotus droebachiensis* (Vasseur, 1949). An increase in respiratory rate has also been reported in *Psammechinus miliaris* (Gray, 1928b; Carter, 1931; Vasseur, 1949). In the same species, egg water was also reported to cause a decrease in sperm respiration (Rothschild, 1952). A decline in respiratory rate was described in *Arbacia punctulata* (Hayashi, 1946), and in *Lytechinus pictus* (Spikes, 1949). In contrast, egg water had no effect on sperm respiration in *S. purpuratus* (Tyler, 1948). It is possible that these varied results were due to differences in the preparation and handling of egg water and spermatozoa before and during the experiments. Rothschild (1956a, 1956b) showed that the rate of sperm respiration was closely dependent on pH, and Mohri and Horiuchi (1961) found that the presence of respiratory CO_2 inhibits the utilization of endogenous phospholipid by spermatozoa, even in a buffered medium.

Experiments on sperm respiration are complicated by the phenomenon known as "Dilution Effect." Gray (1928a) observed that respiration was greater in dilute, than in dense, sperm suspensions, and that dilution of spermatozoa with sea water caused an increase in respiration. It has been suggested that Dilution Effect is due to copper in sea water (Rothschild and Tuft, 1950). This was supported by the demonstration that Dilution Effect is absent when a chelating agent is added to the diluent (Rothschild and Tyler, 1954).

The experiments to be described were initiated after the observation that *Arbacia* egg water caused an increase in sperm respiration, and that the respiratory activator was a substance that could be distinguished from fertilizin. Features of the interaction between the activator and spermatozoa were examined in three species of echinoderms. An electron microscope was used to examine possible changes in the structure of spermatozoa following activation. Some of the results have been described in a preliminary communication (Hathaway, 1960).

Materials and Methods

Arbacia punctulata was collected in the vicinity of the Florida State University Marine Laboratory, Alligator Harbor, Florida, or in some cases, near the Marine Biological Laboratory, Woods Hole, Massachusetts. Other species utilized in Florida were *Lytechinus variegatus* and *Mellita quinquiesperforata*. Eggs and semen were obtained from the gonopores after stimulation by electrical current or 0.55 *M* KCl solution. In both cases the gonads were stimulated directly through an incision in the peroral region. Semen was diluted in five volumes of filtered sea water buffered with glycylglycine, and centrifuged for five minutes at about 5000 *g*. The sedimented spermatozoa were resuspended in buffered sea water to ten times the volume of the semen. Aliquots of this suspension were introduced into the respirometer. Oxygen saturation of this suspension was evidently maintained in the respirometer flasks, since there was no marked increase in O_2 uptake after the additional agitation caused by a simulated tipping. The problem of O_2 saturation in dense suspensions of spermatozoa has been discussed by Rothschild (1956a). Dense suspensions were used in the present experiment to avoid Dilution Effect as far as possible.

Eggs were washed in several times their volume of sea water. Materials were obtained from washed eggs as follows: (1) Washed eggs were allowed to remain three hours in two volumes of sea water. The supernatant is referred to as "egg water." (2) The jelly-coats of the eggs were dissolved with sea water acidified to pH 4.0. The jellyless eggs were carefully washed in several changes of sea water, and then left for three to six hours in five volumes of sea water. The supernatant is referred to as "jellyless egg water." Temperatures were 20–22° C. during the procedures described above. (3) Five ml. of settled eggs were mixed with 25 ml. acidified alcohol (0.004 N HCl in ethanol). The deep red supernatant was dried *in vacuo* at 40° C., mixed with 15 ml. of sea water, and centrifuged. The supernatant was neutralized with NaOH, and the pH was adjusted to 7.99 with buffered sea water.

Glycylglycine at a final concentration of 0.02 *M* was used as a buffer (Tyler and Horowitz, 1937). The effects of chelation by glycylglycine have not been evaluated in this study; however, it would be expected to modify the influence of metals present in sea water. The pH values of solutions were measured with a Beckman Zeromatic meter.

Oxygen consumption was measured in a Warburg apparatus, with air as the gas phase in the flasks. The single-arm vessels of 15 to 20 ml. capacity contained one ml. of sperm suspension in the main compartment, 0.8 ml. of the experimental solution in the side arm, and 0.2 ml. of 10% KOH solution in the center well, which also contained a filter paper wick. Controls were run to obtain values for auto-oxidation, by substituting buffered sea water for the sperm suspension. The flasks were shaken at 120 cycles per minute, with an amplitude of 3.5 cm. Temperature was 20° C. in the Florida experiments, and 25° C. at Woods Hole. The sperm suspensions and the contents of the side arms were adjusted to equal pH values and mixed with buffer before they were placed in the flasks.

RESULTS

Respiratory activation of spermatozoa by egg materials

Results in Table I show that *Arbacia* sperm respiration was initially increased three- to six-fold over controls by *Arbacia* egg water, jellyless egg water, acidic-alcohol extract, and the diffusate from a dialyzed homogenate of eggs. The initially high rates of respiration decreased by the end of the experiment (2½ or 5 hours), but were still higher than control values. Versene had the effect of maintaining a low but constant rate of sperm respiration. There was no auto-oxidation in any of the solutions. Almost constant levels of pH were maintained in the flasks. The slight fall in pH in some flasks was probably caused by the accumulation of CO_2. The results indicate that the increase in sperm respiration is due to agents that diffuse freely from eggs.

Table I

*Oxygen uptake of Arbacia spermatozoa**

Final concentration of glycylglycine = 0.02 *M*.
Sperm concentration = 1/10 × semen.
Initial pH of sperm suspension = 7.99.
Tipped at t = 40 minutes.

Solution tipped in and its pH	Duration of experiment, minutes	Rate of respiration 10 minutes after tipping: μl. O_2/hr.	Rate of respiration at end of experiment, μl.O_2/hr.	Final pH of sperm suspension
Diffusate (dialysate) of homogenized *Arbacia* eggs 7.92	150	565	258	7.83
Acidic-alcohol extract 7.95	150	457	225	7.86
Sea water 7.98	150	92	45	7.98
Versene 2.5×10^{-4} *M* 7.99	150	59	60	8.03
Egg water 7.98	300	286	65	7.90
Jellyless egg water 7.96	300	302	102	7.85
Sea water 8.00	300	100	46	8.00

* Each entry in Tables I–V represents a single determination.

Some properties of activating solutions

The respiratory activator in egg water was lost during dialysis. Whereas undialyzed *Arbacia* egg water caused an immediate increase of 487% in *Arbacia* sperm respiration, the same egg water that had been dialyzed for 24 hours against running sea water had no effect. Although these results may indicate that the active agent is unstable in sea water, the loss of activity could have resulted from diffusion of the activator through the dialyzing membrane. As an additional test a dialysis bag containing 10 ml. of sea water was placed in a beaker containing a suspension of washed *Arbacia* eggs. After six hours at 20°, the contents of the bag and the supernatant sea water (egg water) in the beaker were tested separately for their effects on *Arbacia* spermatozoa. The respiratory rates increased by 254% and 337%, respectively. The diffusion of the activator through dialysis membrane indicates that it is not identical with fertilizin. The activator could be a low molecular weight derivative of fertilizin. If this were the case, the source of activator, like that of fertilizin, would be the jelly-coat of the egg. However, jellyless egg water, which does not agglutinate spermatozoa, is rich in activator, indicating that the source is probably the egg itself, rather than the jelly-coat.

It is known that the agglutination titer of fertilizin is reduced by reaction with spermatozoa. In order to test for a similar reduction in the strength of an activating solution, jellyless egg water was mixed with spermatozoa and left to interact for 90 minutes. The suspension was then centrifuged, and the supernatant solution was tested for activating property. Results in Table II show that the activating property was almost completely lost during the first exposure to sperm, suggesting a rapid absorption of the activator by the cells.

The motility of *Arbacia* spermatozoa was stimulated by a distillable product of *Arbacia* eggs (Clowes and Bachman, 1921). To determine if the respiratory activator has this property, an activating solution that caused an increase in sperm respiration of 987% was boiled under reflux at a pressure of one atmosphere for 20 minutes. The boiled solution stimulated sperm respiration by 950%. A distillate obtained from the activating solution was without stimulating effect on spermatozoa.

Table II

Decrease in the activating property of jellyless egg water during exposure to Arbacia spermatozoa

Solution tipped in	Per cent increase in the rate of O_2 uptake in the sperm suspension, measured 15 minutes after tipping
Jellyless egg water	646
Jellyless egg water exposed to *Arbacia* spermatozoa* for 90 minutes	55
Sea water	10
Sea water exposed to *Arbacia* spermatozoa* for 90 minutes	27

* Spermatozoa washed and diluted in same way as those used in the respirometer.

Jellyless egg water that caused a 917% increase in the rate of O_2 uptake of spermatozoa was extracted with an equal volume of n-butanol, and, after evaporation of the solvent, the residue was taken up in sea water. This solution activated sperm respiration by 750% whereas a butanol extract of sea water had no effect on spermatozoa.

Effects of amino acids upon the rate of sperm respiration

Amino acids and other chelating agents prolong the motility of sea urchin spermatozoa (Tyler, 1953), and stimulate the motility and respiration of starfish spermatozoa (Metz and Birky, 1955). Cysteine can stimulate (10^{-2} M), or depress (10^{-3} and 10^{-4} M) respiration in sea urchin spermatozoa (Mohri, 1956b). It seemed of interest to examine the effects of several amino acids on *Arbacia* spermatozoa. Only cysteine had a marked effect on sperm respiration (Table III), without, however, increasing sperm motility. The magnitude of the response of spermatozoa to cysteine suggested a comparison of some of the properties of the activator and cysteine. Solutions of cysteine at concentrations capable of stimulating sperm respiration yielded colored spots when tested on paper with ninhydrin, but jellyless egg water showed no trace of color when treated in the

TABLE III

Effect of amino acids on Arbacia sperm respiration*

Solution tipped in	Per cent increase in the rate of O_2 uptake by the sperm suspension measured 15 minutes after tipping
Histidine	120
Cysteine	1270
Glycine	53
Glutamic acid	10
Tryptophan	54
Arginine	10
Lysine	43
Sea water	56

* 2×10^{-3} *M* amino acid in buffered sea water.

same manner. Next, jellyless egg water, and a solution of cysteine (4.4×10^{-3} *M*) were each mixed with an equal volume of 4.4×10^{-3} *M* of the sulfhydryl reagent, iodoacetamide. At this concentration, iodoacetamide alone reduced the respiration of spermatozoa to almost nil within 45 minutes. In the mixture with jellyless egg water, iodoacetamide still reduced sperm respiration in 45 minutes, the egg water failing to abolish the inhibitory effect of iodoacetamide. In the mixture with cysteine, however, the inhibitory effect of iodoacetamide was abolished (Table IV). This suggests that the activator in egg water is not cysteine.

In this experiment, the stimulatory effect of cysteine was not diminished by iodoacetamide, and it may be that the sulfhydryl group is not essential for the stimulation of sperm by cysteine. This question was not pursued.

The activator also differs from cysteine in its effect on unbuffered suspensions of spermatozoa of the starfish *Asterias forbesi.* Jellyless egg water from *Arbacia* caused no increase in the respiration of *Asterias* spermatozoa, while 2.2×10^{-2} *M* cysteine caused an increase of 358%. In the same experiment, 2.2×10^{-4} *M* Versene caused an increase of 425% in sperm respiration. The activating action of cysteine in this case was probably a result of its chelating property (Metz and Birky, 1955).

TABLE IV

Effects of Arbacia jellyless egg water, cysteine, and iodoacetamide on the respiration of Arbacia spermatozoa

Final concentration of cysteine and iodoacetamide = 2.2×10^{-3} *M*

Solution tipped in	Respiratory rate before tipping: μl. O_2/hr.	Respiratory rate one hour after tipping; μl. O_2/hr.
Sea water	79	60
Sea water plus iodoacetamide	75	22
Jellyless egg water plus sea water	78	325
Jellyless egg water plus iodoacetamide	78	10
Cysteine plus sea water	82	225
Cysteine plus iodoacetamide	75	250

Species specificity of respiratory activators

Although the agglutination of spermatozoa by egg water is highly specific (Lillie, 1919; Metz, 1945; Tyler, 1948), there has been no clear demonstration of specificity among activators of sperm motility and respiration. A specific activator was thought to occur in starfish egg water (Loeb, 1915), but it was later demonstrated that starfish spermatozoa are virtually immotile in both sea water and egg water (Metz, 1945). In the same paper, Loeb also reported a strong cross-reaction in the activation of sea urchin spermatozoa by egg water. In view of these reports, gametes of several species were examined for respiratory activators.

The non-agglutinating, jellyless egg water from *Lytechinus variegatus* activated the respiration of *Lytechinus* spermatozoa. In a specificity test involving simultaneous observations on *Lytechinus* and *Arbacia* spermatozoa, the activators proved to be species-specific (Fig. 1). This experiment was performed on April 24, 1960. On two other occasions earlier in April, virtually the same results were obtained in similar experiments. A month later, on May 29, 1960, when *Lytechinus* was approaching the height of its reproductive activity, its spermatozoa displayed a strong respiratory response to *Arbacia* activator. However, a complete test of specificity could not be performed at that date.

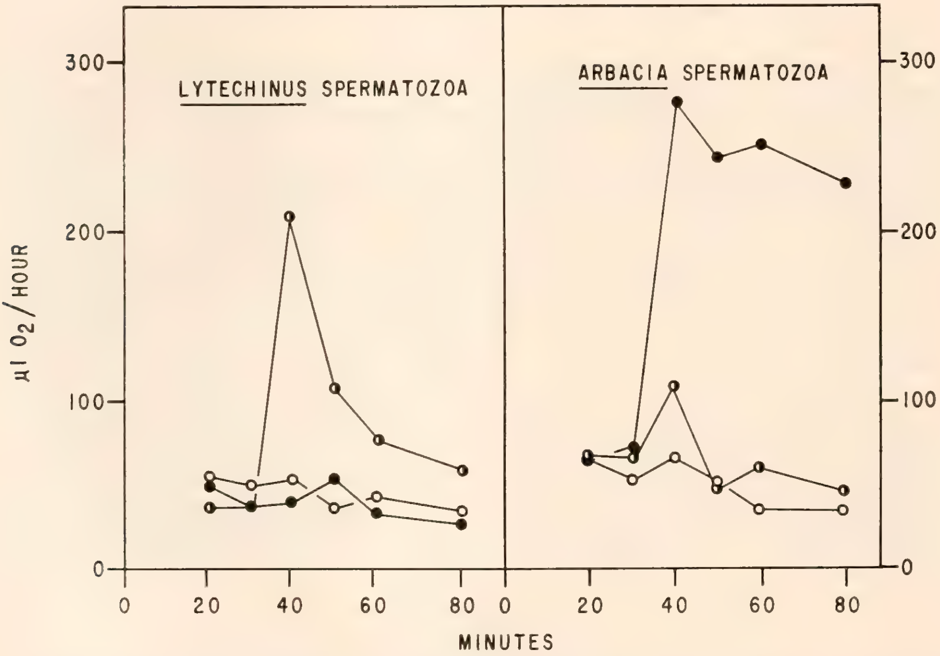

FIGURE 1. Respiratory rates of *Lytechinus* and *Arbacia* spermatozoa in a specificity test of activating solutions. Activating solutions tipped in at $t = 32$ min.: *Arbacia* jellyless egg water ●, *Lytechinus* jellyless egg water ◑, Sea water ○.

The jelly-coats of *Mellita quinquiesperforata* contain numerous red granules which can be liberated with acidified sea water. These granules, when washed and suspended in sea water, stimulated respiration of the spermatozoa of *Mellita* and *Arbacia*. In a simultaneous test, *Arbacia* activator had no effect on *Mellita* spermatozoa (Table V).

Sperm motility

Arbacia spermatozoa, which were moderately active in sea water, became intensely motile when mixed with egg water, jellyless egg water, and acidic-alcohol extract of *Arbacia* eggs, and with suspensions of the jelly-coat granules of *Mellita*. The jelly-coat granules also stimulated the motility of *Mellita* spermatozoa. Concentrations of cysteine that stimulated respiration had no effect on the motility of *Arbacia* sperm. *Lytechinus* spermatozoa were relatively unaffected by solutions prepared from specific eggs or from *Arbacia* eggs.

Other effects of jellyless egg water on spermatozoa

Numerous investigations have demonstrated that sea urchin spermatozoa undergo visible structural changes in the presence of material from eggs. Popa (1927) described the appearance of "lateral bodies" after exposure of *Arbacia* spermatozoa to egg water and vital stains. More recent authors have interpreted the "lateral body" as a displaced middle piece, which appears when spermatozoa are treated with egg water, sea water deficient in calcium, or homogenates of eggs (Tyler, 1952; Dan, 1954; Bernstein, 1959). In addition, echinoderm spermatozoa frequently undergo an acrosome reaction in the presence of egg water (Dan, 1952, 1956; Metz and Morrill, 1955), and *Arbacia* spermatozoa have reacted acrosomes following treatment with purified fertilizin (Piatigorsky and Austin, 1962).

TABLE V

Specificity of activators from Arbacia and Mellita

Solution tipped in	Sperm	Per cent increase in O_2 uptake by the sperm suspension measured 15 minutes after tipping
Arbacia jellyless egg water	*Arbacia* *Mellita*	1660 75
Suspension of jelly-coat granules from *Mellita*	*Arbacia* *Mellita*	1640 285
Sea water	*Arbacia* *Mellita*	10 78

Arbacia spermatozoa were mixed with jellyless egg water, or with sea water, and fixed 20 minutes later in 1% formalin in sea water. Whole-mounts of these cells were examined in an electron microscope at a magnification of 7300. The proportion of displaced middle pieces was small and almost the same in the suspensions treated with activator (12.2% of 465 cells), and in suspensions mixed with sea water (11.4% of 484 cells). Reacted acrosomes were not seen, however; it is common with *Arbacia* spermatozoa to fail in the stimulation of the acrosome reaction by methods (high pH, calcium-free medium, treatment with purified fertilizin) that are usually effective in other species.

Arbacia spermatozoa mixed with egg water release complex materials containing sialic acid (Warren, Hathaway and Flaks, 1960). This phenomenon appears to result from the interaction between spermatozoa and fertilizin, and the evidence suggests that sialic acid is a component of sperm antifertilizin (Hathaway, 1961). It was of some interest, therefore, to observe that *Arbacia* spermatozoa failed to release materials containing sialic acid during the interaction with the respiratory activator in jellyless egg water.

Discussion

The responses of sea urchin spermatozoa to products of eggs are similar in many respects to effects achieved with a variety of other agents of known or unknown composition. The foremost of these effects is an immediate change in oxygen uptake by dense suspensions of spermatozoa, caused by additives ranging from sea water (as in Dilution Effect), and hydrogen ions (pH effect), to uncoupling agents (Rothschild, 1956a), and sulfhydryl reagents (Barron and Goldinger, 1941). In a study of the effects of sodium azide, Mohri (1956a, 1956b) implicated the cytochrome-cytochrome oxidase system in the increased respiration that follows dilution of sperm with sea water, and he showed that the respiratory rate in dilute suspension is equalled in dense suspensions that are supplied with substrates (e.g., dimethyl-p-phenylenediamine) for the cytochrome system. More recently, dithiocarbamates have been found to cause an augmentation of sperm respiration, an effect that is cancelled by the presence of sodium azide (Muramatsu, 1963a, 1963b). Dermal secretions from *Arbacia* stimulate sperm respiration, as well as inhibit fertilization and sperm agglutination (Metz, 1959, 1960, 1961). A similar increase in respiration is found in sperm treated with another inhibitor of fertilization extracted from the alga *Fucus* (Branham and Metz, 1962).

The effect of slight changes in pH on respiration of spermatozoa has been studied by Rothschild (1956a, 1956b). In experiments with *Paracentrotus lividus* and *Echinus esculentus* this author observed a 400% increase in O_2 uptake when the pH value increased from 7.84 to 8.00. Since it is almost impossible to avoid slight changes in pH with dilution, Rothschild warns of the hazard of interpreting respiratory increases as Dilution Effect. This applies equally to interpretations of the effects of various substances on sperm respiration. The situation is different in *Pseudocentrotus depressus*, where spermatozoa undergo only small changes in respiration over a broad range of pH (Mohri and Horiuchi, 1961). These experiments duplicated the conditions used by Rothschild, and it is reasonable to conclude that wide variations exist in the responses of sea urchin spermatozoa to changes in pH.

Several things indicate that the respiratory responses of spermatozoa reported in this paper are not due merely to changes in pH. The pH values of suspensions were not elevated when they were checked at a stage when respiratory rates were still high. In fact, the pH was slightly lower in these suspensions because of the high rate of CO_2 production. Moreover, there was no marked increase in respiration when sea water, rather than egg water, was added, in spite of the fact that it would have been just as difficult to avoid slight changes in pH when adding sea water. The consistent response of spermatozoa to egg materials, and the lack of response to sea water, argue against the interpretation that slight, uncontrollable variations in pH account for the results.

An activator of sperm motility was described in *Arbacia* eggs some time ago (Clowes and Bachman, 1921), but the enhancement of respiration by egg materials has apparently not been observed before in this genus. On the contrary, egg water has been reported to cause a decrease in O_2 uptakes by *Arbacia* spermatozoa (Hayashi, 1946). Hayashi observed a decline in the respiratory rate after addition of egg water to a dilute suspension of spermatozoa. This suspension had already experienced a burst of respiratory activity after dilution with sea water. Under such conditions, O_2 uptake was apparently curtailed by the agglutination of the spermatozoa.

The identity of the respiratory activator, and the mechanism of its action upon spermatozoa, remain unknown. The activator is not identical with fertilizin, since it is diffusible during dialysis, and soluble in acidified ethanol and butanol. Also, it fails to agglutinate spermatozoa, and does not induce the release of sialic acid from these cells. The possibility has not been excluded that the activator is a low molecular weight derivative of fertilizin (*cf.* Tyler, 1955). The fact that this agent is readily obtained from jellyless eggs suggests that it is not simply a breakdown product of fertilizin. This is consistent with the observation that sperm motility is increased by an agent that diffuses from jellyless eggs (Loeb, 1915).

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SUMMARY

1. Sea urchin gametes were used in measurements of the effect of egg materials upon sperm respiration.

2. Dense suspension of spermatozoa of *Arbacia punctulata* had a relatively low rate of oxygen uptake in buffered sea water. After the addition of egg water or materials diffusing from jellyless eggs (jellyless egg water), there was a marked increase in respiratory rates. A similar effect was seen after the addition of cysteine, but not after the addition of several other amino acids, Versene, or buffered sea water. It was concluded that *Arbacia* eggs contain an activator of sperm respiration.

3. Solutions lost their activating properties during interaction with spermatozoa. The respiratory activator diffused through dialyzing membrane, and was alcohol-soluble, heat-stable, and non-volatile. It differed from cysteine in its re-

action with ninhydrin and iodoacetamide. It failed to activate respiration in starfish spermatozoa, unlike cysteine and Versene, which stimulated respiration in these cells. The activating agent in question remains unidentified, but it is evidently neither fertilizin nor cysteine.

4. Respiration of spermatozoa of *Lytechinus variegatus* was increased by diffusates from *Lytechinus* eggs. A species specificity exists in the respiratory activators of *Lytechinus* and *Arbacia.*

5. Suspensions of the red granules from the jelly-coat of *Mellita quinquiesperforata* activated the respiration of spermatozoa of both *Mellita* and *Arbacia.* Diffusates from *Arbacia* eggs failed to affect *Mellita* sperm.

6. No structural changes were observed in *Arbacia* spermatozoa incubated with activating solutions. Unlike fertilizin, the activator in jellyless egg water failed to cause the release of sialic acid from sperm.

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HEMOLYMPH PROTEIN AND COPPER CONCENTRATIONS OF ADULT BLUE CRABS (CALLINECTES SAPIDUS RATHBUN)[1]

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Preparatory to and in conjunction with an investigation of the variability in hemolymph proteins of *Callinectes sapidus* Rathbun, the common Atlantic Coast blue crab, we sought published data on the range and mean values for the protein concentrations in the adults of this species. Although a considerable amount of information is available for many Decapoda, particularly European species, and was conveniently summarized in 1960 by Florkin and by Engle and Woods, little could be found for the blue crab. As our own determinations of hemolymph protein concentrations accumulated, two facts promptly emerged: (1) individual variation of hemolymph protein concentration showed a ten-fold range; and (2) males showed a distinctly lower mean value than females for protein concentration. These findings suggested that considerable caution be exercised in the use of similar published data which for many other species were obtained on a very small sample or on but one individual. Furthermore, the difference in mean values of protein concentration for the two sexes is sufficient to merit attention in future studies directed at determining similar comparative biochemical data.

Wide size variation in the adults of both sexes, already noted by several investigators (Gray and Newcombe, 1938; Tyler and Cargo, 1963), led us to seek possible correlations between body size and hemolymph protein concentration. Finally, because we found in other studies that over one-half of the serum protein was hemocyanin, total copper in individual sera was assayed to provide an indication of which protein components varied sufficiently in their concentrations to produce the wide range in individual total protein concentration observed.

METHODS AND MATERIALS

Data were obtained from 160 female and 98 male adult blue crabs, *Callinectes sapidus,* collected in Core and Bogue Sounds off the coast of Carteret County, North Carolina, during February, March, April and early May of 1963. In addition, 75 female blue crabs "in sponge" taken during May formed the basis for a third group of data.

During the first two months of this study, hemolymph was collected from crabs shipped on ice to Durham from the Duke University Marine Laboratory. Although these specimens were usually quite active on arrival and were bled immediately, the wide variability in their individual serum protein concentrations suggested that some individuals might have been affected by the conditions under which they were transported. Hence, all further analyses (all sponge females and over one-half of the remainder of the sample) were made on serum obtained at the Marine Laboratory either from animals just taken from the field or within twelve hours from animals held in well-aerated tanks of running sea water. Some of the latter groups and some sponge females were tagged, kept in the tanks more than a week, fed *ad libitum,* and bled at intervals of three to four days to follow the serum protein and total copper levels of individuals through this period. Forty-one male crabs bled consecutively were divided into two groups, dependent upon the total amount of hemolymph removed at each bleeding. From 22, <5 ml. per bleeding were withdrawn; from 19, >5 ml. per bleeding were withdrawn.

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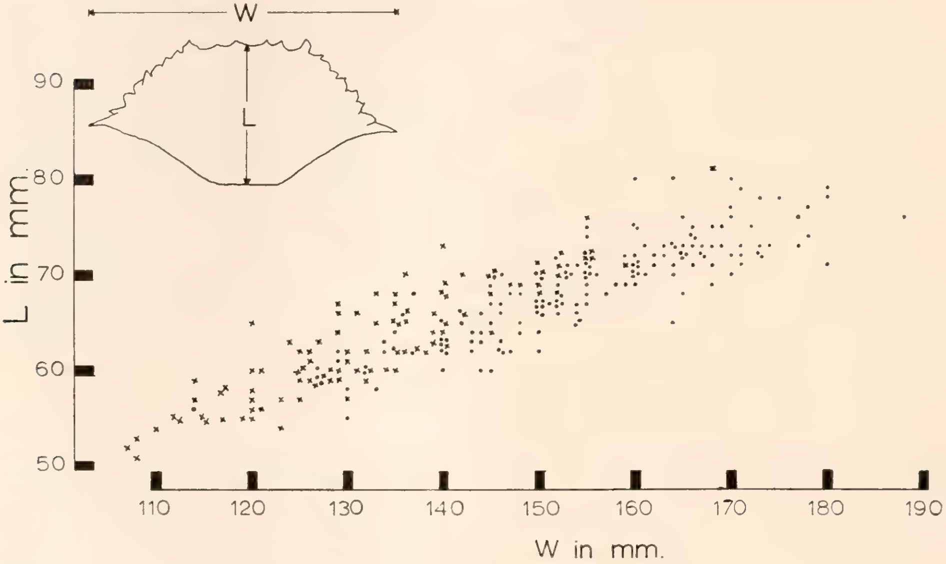

FIGURE 1. Length-width values of 98 male (x) and 160 female (●) adult specimens of *Callinectes sapidus*. Means: ♂L = 61.17; ♀L = 63.79; ♂W = 132.55; ♀W = 155.18.

Measurements on each crab were recorded for the shortest distance between the ends of the lateral carapace spines, the *width* (W), and for the distance from the median anterior groove, just behind the rostrum, to the posterior edge of the carapace, the *length* (L) (see diagram on Figure 1). Appendage defects and losses were noted for each individual.

Hemolymph was withdrawn by syringe from the sinuses at the base of the chelae and swimming legs, and allowed to clot in individual refrigerated centrifuge tubes. Clots were broken with a glass rod and squeezed out by centrifugation. The individual sera were separated from the clots and stored at 4° C.

The protein content of each serum was determined by the method of Lowry *et al.* (1951), standardized with crystalline bovine serum albumin and with lyophilized hemocyanin. The standard curves for the two proteins were indistinguishable.

Total copper concentration was determined by the 2,2′-biquinoline reaction of Klotz and Klotz (1955), modified by the addition of cysteine hydrochloride as suggested by Felsenfeld (1960). The test was standardized against both copper sulfate and against copper foil with no detectable difference in the concentrations used.

Large pools of adult male and female crab sera were dialyzed in the cold (4° C.) against repeated changes of distilled water over five to seven days. After centrifugation at an RCF of 750 for 30 minutes, the clear blue supernatant fluid was lyophilized and stored at −20° C.

RESULTS

Size

Figure 1 is a plot of the length-width relationships for the 98 male and 160 female crabs. Interestingly, this small sample of the North Carolina blue crab population south of Cape Hatteras proved remarkably similar in length-width relationship to the adult blue crab population sampled by Gray and Newcombe (1938) from Chesapeake Bay. Using our mean value for width in their equations derived to express the relationship between the linear dimensions, length and width, the predicted value for mean length of females was within 1% of the observed mean value for that dimension; of males within 3%. Table I summarizes size data for all three groups of adults. Generally, in the same width-class males tend to be longer than females. A test of the significance of the difference between the mean width of

TABLE I

Summary of morphometric and chemical data

Number of individuals	Males 98	Females 160	Sponge females 75
Length (mm.):			
Range	51–76	50–75	50–74
Mean	61.17	63.79	62.08
Standard deviation	5.01	5.26	5.39
Width (mm.):			
Range	107–168	115–188	119–188
Mean	132.55	155.18	151.92
Standard deviation	12.89	13.53	15.23
W/L Index:			
Range	1.8462–2.5000	2.1333–3.0508	2.109–2.692
Mean	2.1666	2.4343	2.4461
Standard deviation	0.1105	0.1179	0.0994
Serum protein (mg./ml.):			
Range	8.8–132	15.2–119.2	40–111
Mean	52.37	62.70	75.82
Standard deviation	24.47	17.15	17.77
Serum copper (μg./ml.):			
Range	8–173	16–176	43–165
Mean	70.65	83.86	93.15
Standard deviation	34.39	28.83	26.48
Copper-protein ratio:			
Range	0.0545–0.1900	0.0568–0.1982	0.070–0.170
Mean	0.1358	0.1341	0.1230
Standard deviation	0.0256	0.0284	0.0195

males and that of females showed the probability of exceeding the observed value of *t* (13.2739) to be much smaller than 0.001. A similar test of the W/L index for males and females showed the probability of exceeding the observed value of *t* (18.1357) also to be much smaller than 0.001. The differences in the compared means are, therefore, highly significant.

Serum protein and copper

The individual determinations of serum protein concentration in mg./ml. and total copper in μg./ml. are shown in Figure 2, together with the means for both values for both sexes. The line has been drawn through points which represent a copper/protein ratio ×100 (C/P ratio) of 0.200, a probable upper limit for the copper/protein ratio of pure hemocyanin from blue crab hemolymph.

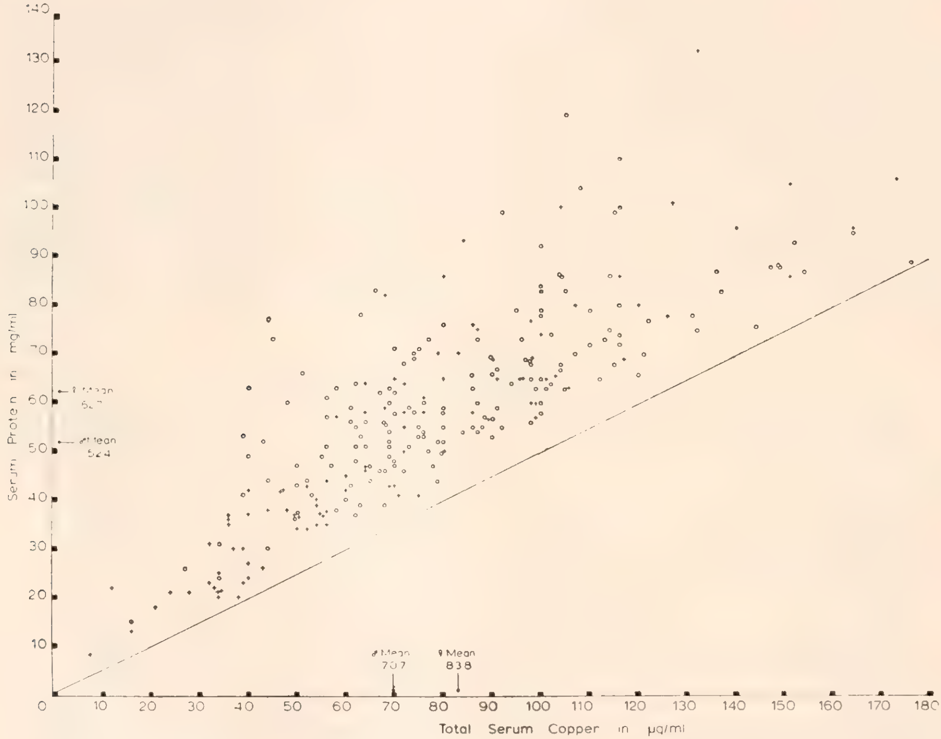

FIGURE 2. Individual serum protein and serum total copper values for 98 male (+) and 160 female (o) adult specimens of *Callinectes sapidus*. The solid line represents points calculated for protein with a copper/protein ratio (× 100) of 0.200.

No correlation could be found between crab size and copper or protein values. Table I summarizes data on mean serum protein concentrations, mean total serum copper concentrations and mean C/P ratios for all three groups. Given below are tests for the significance of the difference between the various means observed, together with the probability that *t* will fall outside these values:

Protein of ♂ compared with protein of ♀: $t = 3.9800$, <0.001
Protein of ♀ compared with protein of sponge ♀: $t = 5.4059$, <0.001
Copper of ♂ compared with copper of ♀: $t = 3.3148$, <0.001
Copper of ♀ compared with copper of sponge ♀: $t = 2.3623$, 0.01–0.02
C/P ratio of ♂ compared with C/P ratio of ♀: $t = 0.4899$, 0.6–0.7
C/P ratio of ♀ compared with C/P ratio of sponge ♀: $t = 3.0714$, 0.001–0.01

Thus, there is a significant difference between all mean values compared except between mean C/P ratios of male and non-sponge bearing female crabs.

No correlation could be discovered between individual serum protein concentration and the absence of or defects in appendages in individual adults of both sexes.

Consecutive bleeding experiments

A total of 41 marked adult male crabs were bled in the described manner, from 22 of which less than 5 ml. (usually 3.0 ml.) of hemolymph were withdrawn and from 19 more than 5 ml. were withdrawn. Four days later hemolymph was withdrawn in the same amounts from the same animals. This procedure was repeated at three more four-day intervals. At the third bleeding (day 8) only 22 animals had survived, 14 bled <5 ml. (64% survival), 8 bled >5 ml. (42% survival). A comparison of mean total protein and copper concentrations, together with mean C/P ratios for the hemolymphs sampled at days 0 and 8 in both groups of animals, is made in Table II. Survival was too poor for reasonable comparisons after day 8. The t-tests for the differences between paired samples (day 0 and day 8) were computed for both sets of individuals for protein and copper concentration and for C/P ratios. The results were as follows:

TABLE II

Changes in blue crab serum protein and copper concentrations in consecutive bleeding experiment (see text). All values are means with standard deviation. Means of C/P ratios are means of individual ratios

	<5 ml./bleeding; 14 animals		>5 ml./bleeding; 8 animals	
	Day 0	Day 8	Day 0	Day 8
Mean protein concn. (mg./ml.)	60.51	45.17	43.05	33.70
Stand. dev.	28.74	14.19	22.65	10.17
Mean copper concn. (μg./ml.)	75.00	61.64	48.50	50.75
Stand. dev.	28.41	14.61	22.87	14.30
Mean C/P ratio × 100	0.1310	0.1412	0.1160	0.1517
Stand. dev.	0.0292	0.0219	0.0173	0.0102

Crabs bled <5 ml./bleeding:

Protein (day 0 and day 8) $t = 2.8373$, 0.01–0.02
Copper (day 0 and day 8) $t = 2.5171$, 0.02–0.05
C/P (day 0 and day 8) $t = 1.4657$, 0.1–0.2

Crabs bled >5 ml./bleeding:

Protein (day 0 and day 8) $t = 1.8437$, 0.1–0.2
Copper (day 0 and day 8) $t = 0.6094$, 0.5–0.6
C/P (day 0 and day 8) $t = 5.7774$, 0.01–0.001

In another experiment of similar design 10 sponge females were bled 5 ml. each on days 0, 3, and 6. All survived analyses of the same parameters as in the above experiment and yielded the following results:

	Day 0	*Day 6*
Mean protein concn. (mg./ml.)	81.64	64.45
Mean copper concn. (μg./ml.)	103.7	87.5
Mean C/P ratio	0.127	0.136

The results are essentially similar to those of the preceding experiment. There was a decrease in protein concentration of about 20%; a less drastic change in copper concentration; and a consequent increase in C/P ratio.

Discussion

Although the sample of blue crabs used in this study was relatively small, comparison of size data with those obtained by Gray and Newcombe (1938) from blue crabs in Chesapeake Bay shows good correspondence in the width-length relationships which were expressed mathematically for both sexes. Tyler and Cargo (1963), in their comparative study of penultimate and ultimate instars of female blue crabs from different portions of Chesapeake Bay, presented data on over 2000 crabs which support the wide morphometric variation this species exhibits. Their findings also support older reports of groupings (or schools) of crabs which vary significantly in size from locality to locality or in the same locality with time. We have not detected a significant variation in the samples used in this study during the period collected. In any case, the total sample of crabs in this study shows sufficient variation in morphometry and sufficient correspondence with mean dimensions and width-length relationships already published for other blue crab populations that it may be assumed to be closely representative of the population of such crabs for the region and season in which taken.

No published data on the hemolymph protein concentration of the blue crab have been found. Summaries of comparable data for other Decapoda (Florkin, 1960; Engle and Woods, 1960) reveal ranges and mean values of protein concentration for many species based on less than a dozen specimens. There appear to be few data based on a sufficient number of specimens with which a meaningful comparison can be made either for mean serum protein concentrations or their ranges according to sex.

The analysis of 41 individual serum protein concentrations in *Maia squinado* by Drach and Teissier (1939) showed three- to four-fold differences. The same individuals after molt, despite an abrupt drop in protein concentration, showed the same range in individual variation. Webb (1940), in his study of ionic regulation in *Carcinus maenas*, gives determinations of plasma protein concentration in six pools representing up to 18 animals of undesignated sex. The range was from 43 to 72 mg./ml. of protein with a mean of 60 mg./ml. These values were admittedly approximations, having been obtained by subtracting the weight of salts from the weight of total solids, but nevertheless suggest a wide range of concentrations in this species also.

Drilhon-Courtois (1934) included in his data on mineral composition of *Carcinus maenas* hemolymph a value for total protein in males of 4.0% and in females 4.26%. These data were obtained from pooled hemolymph collected from undesignated numbers of individuals. Protein was determined by weight difference after precipitation and drying. These are the only published figures, of which we are aware, comparing male with female hemolymph protein concentrations.

Allison and Cole (1940) concluded that, after clotting, the sera of *Homarus americanus, Cancer borealis* and *Callinectes sapidus* contained only hemocyanin (HCy) and that the concentration of this protein varied as much as 100% in different individuals. Unfortunately, no data were presented to substantiate individual variability in *Callinectes sapidus.* It is doubtful from our own unpublished work that the isolation methods used by these investigators would exclude apohemocyanin from the blue crab product they analyzed, thus decreasing to some unknown degree the Cu:N ratio they obtained for the hemocyanin of this species.

The ten-fold range in individual serum protein concentrations could not be correlated with size, with the number or condition of appendages, or with the immediate previous history of the animals, *i.e.,* shipped over ice to Durham, fresh from the field, or held in tanks of running sea water.

The degree of variability in copper-bearing protein (principally hemocyanin) and non-copper-bearing protein (principally apohemocyanin) concentrations is shown in Figure 2. Those individuals in this Figure designated by points close to the line representing a C/P ratio of 0.200 should possess sera having a very high proportion of their total protein as HCy. Starch gel electrophoretic patterns of the sera from such individuals confirm this prediction. Individuals whose serum protein and/or serum copper concentrations place them far from the line possess sera which produce quite different patterns of band concentration after electrophoresis. These observations parallel those already made by Manwell and Baker (1963) in their study of the heterogeneity of HCy from *Callinectes sapidus* when subjected to electrophoresis on starch gel.

A comparison of the mean values for serum protein concentration, serum copper and C/P ratios between male and female crabs shows generally lower values for the protein and copper of male sera but essentially the same C/P ratio. If one assumes a value of 0.200 for the C/P ratio of pure HCy, and if one assumes that HCy is the only copper contributor, then, on the average, 68–69% of male and female serum proteins are HCy, the bulk of the remaining protein being apohemocyanin.

The data which have been obtained from the sera of sponge females bear special attention (see Table I). The mean concentration of serum protein is considerably higher than that obtained for non-sponge females (75.8 mg./ml. vs. 62.7 mg./ml.); so too is the total serum copper (93.2 μg./ml. vs. 83.9 μg./ml.). The differences in these increments of unit protein and copper are such, however, that the mean C/P ratio is considerably lower for sponge females. Without further analysis it might be presumed that simultaneous increases in concentrations of HCy and of non-copper-bearing proteins have occurred. If the same assumptions as made above hold true, the sera of sponge females contain on the average about 63% HCy.

The consecutive bleeding experiments (see Table II *et seq.*) revealed an expected and consistent pattern of protein loss (between 20 and 25%, regardless of amount taken) over the first week of bleeding. This decrease in protein concentration was accompanied by only minor changes in copper concentration, resulting in an increased C/P ratio in all groups.

To account for these changes one might assume HCy synthesis with little or no concomitant production of new non-copper-bearing hemolymph proteins. However, we think a more likely explanation is the conversion of apohemocyanin to HCy by the addition of copper from some stored source such as in the hepatopancreas, an organ rich in copper in the spidercrab, *Maia* (Zuckerkandl, 1957), terrestrial isopods (Wieser and Makart, 1961), and *Octopus* (Ghiretti-Magaldi *et al.*, 1958).

All of these studies are being extended by regular collections and determinations to discover possible seasonal differences. Changes at molting are also being investigated. In addition, electrophoretic and serological analyses of all individual sera are in progress.

We are particularly indebted to Miss Judith Payne for securing and shipping us the live specimens used in the early portion of this study, and to the personnel of the Duke University Marine Laboratory for their generous provision of tank space and laboratory facilities on short notice. The statistical analysis was immeasurably simplified by the Duke University Digital Computing Laboratory, using the TSAR (tape storage and retrieval) System designed and programmed by Mr. Robert D. Carlitz and Mrs. A. J. Gabor.

SUMMARY

1. Width, length and the concentrations of hemolymph protein and copper were determined individually for 333 adult blue crabs, *Callinectes sapidus* Rathbun. Of these, 160 were adult females, 98 adult males, and 75 females in sponge.

2. In the same width-class males tend to be longer than females.

3. Wide variation in serum protein and copper concentrations was found in the three groups. There was no correlation between specimen size and mean serum protein or copper concentrations. Mean serum protein and copper concentrations of males were lower by a significant difference than those for all females; sponge females exhibited correspondingly higher mean serum protein and copper concentrations than adult females without sponge. The mean copper-protein ratios of adult male and female crab hemolymph were essentially identical and higher by a significant difference than that value for the hemolymph from sponge females.

4. Crabs bled at regular intervals exhibited a decrease in mean serum protein levels and an increment in the mean copper-protein ratios. An explanation of this observation is discussed.

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MARINE BIOLO

MORPHOLOGICAL EFFECTS OF COBALTOUS CHLORIDE ON THE DEVELOPMENT OF LIMNAEA STAGNALIS AND LIMNAEA PALUSTRIS[1]

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Numerous investigators have reported the effects of altering the environment of developing embryos, especially echinoderms, with divalent ions of cobalt, nickel and zinc, the transition metals. Echinoderm eggs exhibit hypodevelopment of the endoderm and mesoderm after treatment with these ions. Exogastrulae and abnormal pluteus larvae with reduced arms, skeletal rods, and gut commonly result (Waterman, 1937; Rulon, 1953, 1955, 1956, 1957; Lallier, 1955a, 1955b, 1956, 1959; Mateyko, 1961). A pronounced animal-vegetal gradient and a developmental requirement for a salt-containing medium characterize the echinoderm egg. A mosaic egg, capable of development in a de-ionized environment, might be expected to respond differently to transition metals. In the absence of published information concerning the effect of transition metals on such eggs, a study was made of the morphogenetic effects of one transition metal, cobalt, on the egg of *Limnaea,* a fresh-water pulmonate snail.

MATERIALS AND METHODS

The initial experiments were performed in Utrecht, the Netherlands, with eggs of *L. stagnalis;* in the latter experiments, eggs of the American species, *L. palustris,* were used.

Encapsuled eggs were treated according to the standard conditions of Raven (1956) and Geilenkirchen (1961), to permit comparison with previous studies on *Limnaea.* When the eggs reached the two-cell stage (stages 3 to 6 of Raven, 1946), the egg capsules were freed from the surrounding jelly and divided into lots of 20. The 24-hour treatment was begun at this stage or 24 or 48 hours later. During the treatment period the eggs were incubated at 25° C. in shell vials with various concentrations of $CoCl_2$ in 10 ml. of distilled water. Control eggs were treated similarly but incubated in pond water or distilled water of pH 6–7. Eggs treated during the later periods developed first in tap or pond water or on 2% agar plates.

Embryonic development was studied from whole mounts and serial sections made at 24-hour intervals from the end of the treatment until the normal embryos were larval snails (6–8 days after oviposition). Whole mounts were prepared of embryos fixed in Bouin's solution and cleared in wintergreen oil. Serial sec-

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tions were prepared of embryos fixed in Bouin's solution, sectioned at 6–8 micra and stained with Ehrlich's or Heidenhain's hematoxylin and eosin.

RESULTS

The morphological effects of 24-hour treatment of *Limnaea* eggs or embryos with $CoCl_2$ (1×10^{-4} *M* to 2×10^{-6} *M*) are given in Table I (*L. stagnalis*) and Table II (*L. palustris*). The histological and morphological characteristics of these malformations are described in subsequent sections.

Table I summarizes the results of ten experiments on eggs of *L. stagnalis* treated during the first 24 hours of development and three experiments on embryos treated during the second (gastrula to trochophore stage of Raven, 1946) and third (late trochophore to early veliger stage of Raven, 1946) 24-hour period. Concentrations of $CoCl_2$ greater than 10^{-4} *M* killed the embryos directly; at 10^{-4} *M* few survived the treatment period and none developed to the snail stage ("hippo" stage of Raven, 1949). Treatment with lower concentrations of $CoCl_2$ (8×10^{-5} to 1×10^{-5} *M*) during the first 24-hour period reduced direct mortality and increased the numbers of normal snails. The incidence of exogastrulae and arrested gastrulae was approximately constant at all but the lowest concentration tested; arrested veligers and shell-less snails were most frequent with 4×10^{-5} *M* $CoCl_2$. None of the embryos treated during the second and third 24 hours developed beyond the veliger stage. Those which were not killed during the treatment

TABLE I

Morphological effects of cobaltous chloride on the development of eggs and embryos of Limnaea stagnalis

Treatment		No. of eggs	Effect of Treatment					
Treatment period	Conc. $CoCl_2$ (*M*)		Direct mortality %	Exogastrula %	Gastrula %	Veliger %	Snail	
							Shell malformations %	Normal %
First 24 hours	1×10^{-4}	166	82.5	11.4	4.8	1.3	—	—
	8×10^{-5}	160	64.4	9.4	10.6	14.4	—	1.2
	4×10^{-5}	325	29.5	7.7	11.7	39.8	10.5	0.8
	3×10^{-5}	179	21.2	16.2	12.9	30.8	4.9	14.0
	1×10^{-5}	196	10.2	6.6	1.5	6.6	8.7	66.4
	0	288	2.0	—	—	—	—	98.0
Second 24 hours	1×10^{-4}	60	65.0	—	23.0	8.0	-	—
	5×10^{-5}	60	35.0	—	61.0	4.0	-	—
	1×10^{-5}	60	21.6	—	—	78.4	-	—
	0	60	3.3	—	—	—	—	96.7
Third 24 hours	1×10^{-4}	57	90.0	—	10.0	—	—	—
	5×10^{-5}	57	86.0	—	10.0	4.0	—	—
	1×10^{-5}	57	51.0	—	1.0	49.0	—	—
	0	60	—	—	—	—	—	100.0

period were arrested at the pre-veliger stage or developed into typical arrested veligers with reduced larval liver, foot and shell. The reduction of $CoCl_2$ concentration from 5×10^{-5} M to 1×10^{-5} M increased ten-fold the incidence of arrested veligers among the group treated during the second 24 hours. It appears that this method might be used to produce arrested veligers selectively, as the yield approached 80%. The results in Table I suggest that *L. stagnalis* embryos may be more sensitive to cobalt during the third than during the first or second 24 hours of development.

TABLE II

Morphological effects of cobaltous chloride on the development of eggs and embryos of Limnaea palustris

Treatment		No. of eggs	Effect of Treatment						
Treatment period	Conc. $CoCl_2(M)$		Direct mortality %	Exogastrula %	Gastrula %	Veliger %	Snail		
							Shell-less %	Helmet %	Normal %
First 24 hours	8×10^{-5}	200	81.0	12.0	5.0	2.0	—	—	—
	4×10^{-5}	200	33.5	55.0	5.0	5.0	1.0	–	—
	2×10^{-5}	120	4.2	71.5	3.3	15.8	3.3	0.8	—
	1×10^{-5}	200	12.5	20.5	7.0	29.5	17.5	0.5	9.5
	6×10^{-6}	170	4.7	4.7	—	18.8	24.2	21.2	26.4
	2×10^{-6}	120	2.5	—	—	5.0	20.0	7.5	65.0
	0	200	—	—	—	—	—	1.5	98.5
Second 24 hours	8×10^{-5}	120	93.3	—	—	6.7	—	—	—
	4×10^{-5}	120	81.7	—	—	15.0	3.3	—	—
	1×10^{-5}	120	65.0	—	—	21.3	13.3	—	—
	6×10^{-6}	120	6.7	—	—	6.7	45.0	6.7	33.9
	2×10^{-6}	120	2.1	—	—	3.0	13.3	5.0	76.6
	0	120	—	—	—	—	–	—	100.0
Third 24 hours	8×10^{-5}	120	90.0	—	—	6.6	—	3.4	—
	4×10^{-5}	120	75.0	—	–	—	10.0	—	15.0
	1×10^{-5}	120	13.4	·	—	5.0	10.0	—	71.6
	6×10^{-6}	120	6.6	—	—	—	—	13.4	80.0
	2×10^{-6}	120	16.7	—	—	—	—	—	83.3
	0	120	10.0	—	—		3.4	—	86.6

Table II summarizes the results of ten experiments on *L. palustris* embryos treated during the first 24 hours and six experiments on embryos treated during the second (trochophore to early veliger stage) and third (early to mid-veliger stage) 24 hours of development. The effective concentrations of $CoCl_2$ ranged from 2×10^{-6} to 8×10^{-5} M. Concentrations greater than 4×10^{-5} M killed most of the eggs. The maximal percentage of exogastrulae resulted when embryos were treated during the first 24 hours with 2×10^{-5} M cobalt. The maximum number of arrested veligers and shell-less snails was obtained following treatments during the first or second 24 hours with 10^{-5} and 6×10^{-6} M cobalt. Exogastrulae and arrested gastrulae were absent in embryos treated after the first 24 hours, since the embryos had gastrulated by then. In contrast to the results with *L. stagnalis, L. palustris* embryos were most resistant to exposure to $CoCl_2$ during the third day of development, since normal snails were obtained following treatment with concentrations as high as 4×10^{-5} *M*. During the first 24 hours *L. stagnalis* appeared to be more resistant than *L. palustris*, but the situation was reversed during the second and third periods. These differences are probably due to differences in rate of development; *L. palustris* develops faster than *L. stagnalis* and may have passed the sensitive stages by the third 24 hours. There does not appear to be any critical period of cobalt sensitivity.

Both species of eggs varied considerably in resistance from one experiment to the next and within one experiment. Seasonal changes in the physiological sensitivity (Haije and Raven, 1953; Raven, 1956; Geilenkirchen and Nijenhuis, 1959) cannot fully explain the variations in sensitivity in the present study, as they were observed also among eggs from the same egg mass.

Morphology of malformations

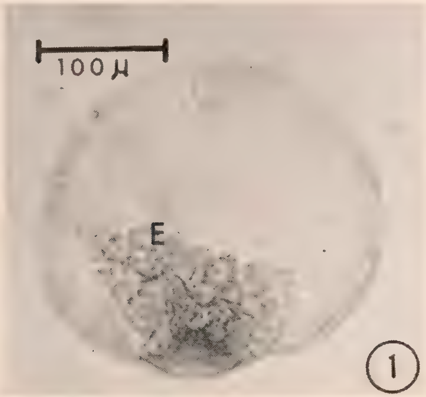
Seven grades of developmental disturbances, ranging from death to slowed normal development, were observed in treated embryos of *L. stagnalis* and *L. palustris*.

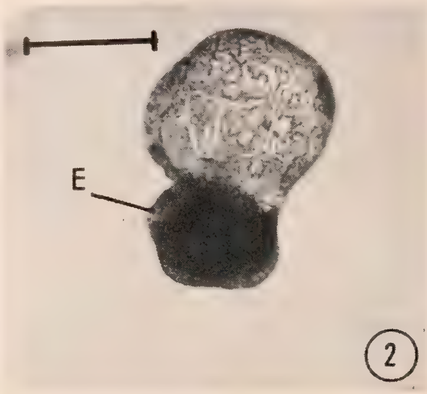
Direct mortality. This category includes embryos which died during the treatment period. Eggs killed during the first 24 hours were usually arrested in the early cleavage and pre-gastrula stages. The blastomeres tended to be spherical and to separate from one another, especially at the eight-cell stage, where the four macromeres often had separated from one another but remained attached to the first quartette of micromeres. This condition suggests a preferential loss of cell adhesiveness at the vegetal pole of the embryo. In several experiments the separated macromeres of *L. palustris* eggs treated with 4×10^{-5} *M* $CoCl_2$ were the same size as the micromeres, indicating that the third cleavage plane had been depressed toward the vegetal pole. The cells of embryos killed during the second and third 24-hour treatment periods also tended to be spherical and to separate from one another.

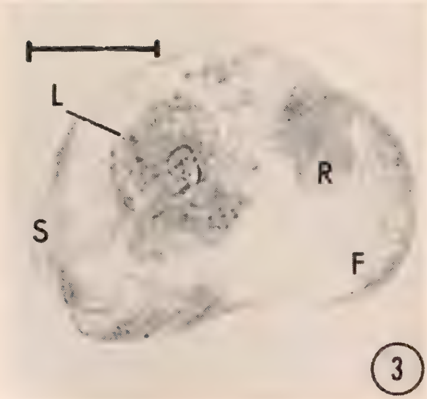
Exogastrulae. Embryos arrested at the gastrula stage frequently developed into vesicular exogastrulae with subpressed invagination of the archenteron (Raven, 1942). They often swelled into hydropic vesicles with a group of endodermal cells visible in the blastopore region (Fig. 1). Such vesicles usually died within a few days, but an occasional one lived as long as 10 days. Dumbbell-shaped exogastrulae (Fig. 2) occurred less frequently. Occasionally a dumbbell-shaped exogastrula would separate into two vesicles; neither developed further.

Arrested gastrulae. This group includes embryos that gastrulated but were one-half to two-thirds the size of the control gastrulae. A few developed as far as the trochophore stage of Raven (1946); none developed into veligers.

Arrested veligers. The most conspicuous morphological malformation occurred at the veliger stage (Raven, 1946), when the normal 4-day embryo or veliger (Fig. 6) had the following features: a distinct head, foot, and body region; paired cephalic plates; a stomodeum and radula sac; a shell and mantle fold covering most of the body; and a body lined with a cup-shaped mass of large endoderm cells. The large endoderm cells formed the larval liver or digestive gland and surrounded the small endoderm cells of the developing mid-gut. The cobalt-arrested veligers (Figs. 3, 4, 5) were smaller than the normal 4-day veliger. Their shells were limited to the area of the shell gland primordium on the left posterior side of the embryo; the larval liver cells formed a compact mass of cells separated from the body wall by a fluid-filled space that extended into a rudimentary foot. The stomodeum, radula sac (Fig. 3) and paired cephalic plates usually were visible. Some arrested veligers became hydropic, showing a swollen foot or body. The vesicular condition of the body apparently resulted from a reduction in the number and size of the large endoderm cells. The vesicular foot was associated with a reduction in mesenchyme tissue. Arrested veligers usually lived for 3–6 days without undergoing further differentiation. The cobalt-arrested veligers were similar morphologically to "aspecific snails" obtained from







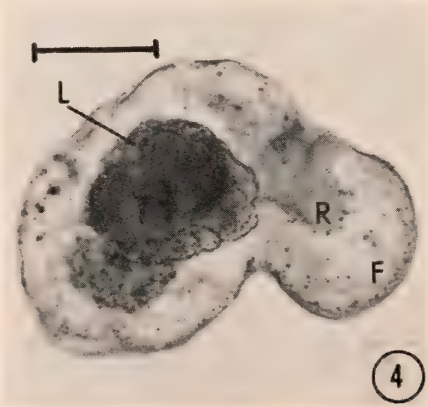


FIGURE 1. Whole mount of an 8-day-old hydropic exogastrula with invaginated mass of endoderm cells.

FIGURE 2. Whole mount of a 4-day-old dumbbell-shaped exogastrula with invaginated endoderm.

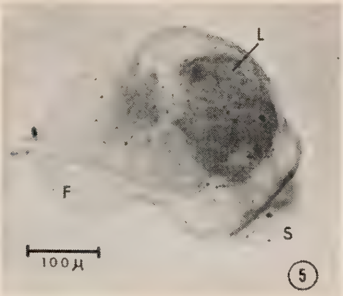
FIGURES 3 and 4. Whole mounts of 8-day-old cobalt-arrested veligers with reduced larval liver mass.

Key for Figures 1 to 4. E, endoderm; F, foot; L, larval liver; R, radula sac; S, shell gland.

centrifuged eggs (Raven and Tates, 1961) and to embryos classified under "other malformations" obtained by lithium treatment (Geilenkirchen and Nijenhuis, 1959).

Shell-less snails. These embryos developed as far as the hippo stage (Raven, 1942), had a pulsating heart and paired eyes and tentacles. They were usually smaller than the control embryos and had the following disturbances: a shell limited to the shell gland area, a relatively small mass of larval liver cells in the center of the body, an occasional hydropic foot or body. These snails never hatched from their capsules.

Helmet-shelled snails. A few embryos developed into snails that were normal except that the shell had an abnormally wide aperture. These resembled the helmet-shelled snails Raven and Spronk (1952) produced with beryllium chloride treatment.



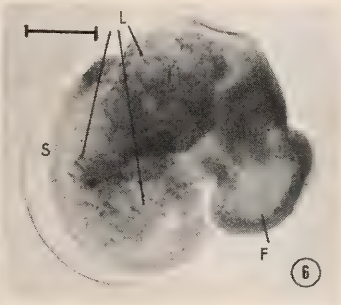
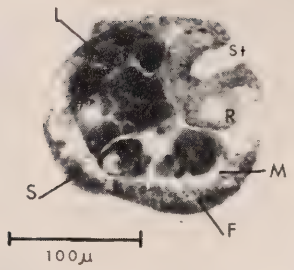


FIGURE 5. Whole mount of an 8-day-old cobalt-arrested veliger.
FIGURE 6. Whole mount of a normal 4-day-old veliger.
Key for Figures 5 and 6. F, foot; L, larval liver; S, shell gland and shell.

Normal snails. Snails which developed from this group were morphologically similar to the controls, although in most cases they developed more slowly.

Histological malformations

Arrested and normal veligers. Histological sections were prepared of normal 3- and 4-day embryos and of 8-day arrested veligers of *L. stagnalis*, to determine the extent of endo-mesodermal differentiation in the cobalt-arrested veligers. The normal 3-day *L. stagnalis* embryo possessed the shell gland, stomodeum and radula sac (Fig. 7). The large endoderm cells of the larval liver were numerous, densely eosinophilic, and closely juxtaposed to the ectoderm, occupying most of the body cavity. At this stage the foot was only a bulge ventral to the stomodeum. Sections of the normal 4-day *L. stagnalis* embryo (Fig. 8) revealed that the larval liver cells lined most of the body proper and were arranged in one to two cup-shaped layers surrounding the mid-gut. The small-celled endoderm was differentiated into mid- and hind-gut. The shell gland had developed into the mantle fold surrounding the posterior half of the body. The foot was distinct and filled with mesenchyme cells. The tentacle *anlagen* were present. Comparison of Figures 7 and 8 shows that the 4-day embryo is almost twice as large as the



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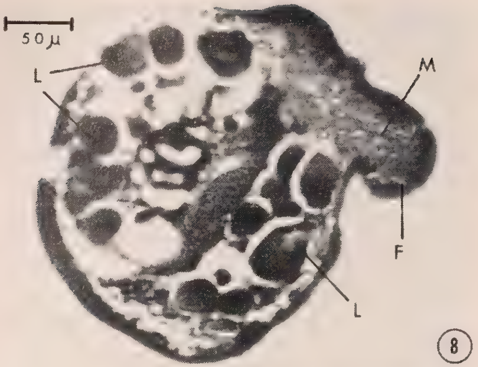

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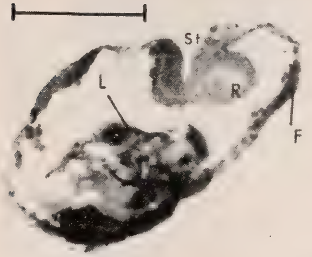

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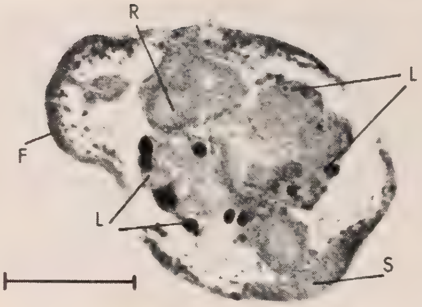

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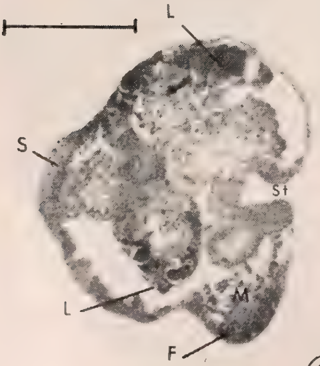

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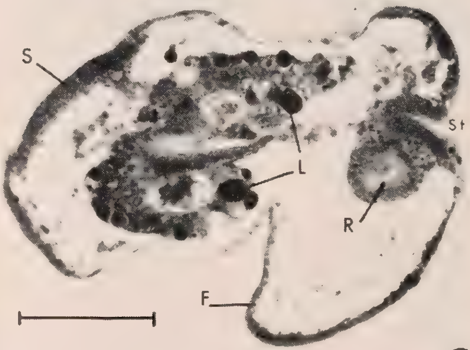

(12)

FIGURE 7. Sagittal section of a normal 3-day-old embryo or late "trochophore."

FIGURE 8. Sagittal section of a normal 4-day-old embryo or "veliger."

FIGURE 9. Sagittal section of an 8-day-old arrested veliger treated the first 24 hours with 4×10^{-5} M $CoCl_2$.

FIGURE 10. Sagittal section of an 8-day-old arrested veliger treated the first 24 hours with 4×10^{-5} M $CoCl_2$.

FIGURE 11. Sagittal section of an 7-day-old arrested veliger treated the first 24 hours with 3×10^{-5} M $CoCl_2$.

FIGURE 12. Sagittal section of an 8-day-old arrested hydropic veliger treated the first 24

3-day embryo, indicating that during the third day of development there is considerable growth accompanying the initial phases of organogenesis.

Sections of 8-day arrested veligers of *L. stagnalis* (Figs. 9, 10, 11) revealed that the stomodeum and radula sac were similar to those of the normal 3-day embryo, and that the small-celled endoderm had formed a mid-gut that in some embryos was continuous with the stomodeum. Therefore, these embryos were morphologically equipped to ingest nutrients from the surrounding capsule fluid. However, the larval liver cells, the main site of capsule fluid digestion at this stage (Bloch, 1938; Raven, 1958), were abnormally small, few in number, and irregularly eosinophilic (Figs. 9–12). The relatively large space between the larval liver and body wall and the foot contained few mesenchyme cells; the hydropic veligers were especially deficient in mesenchyme cells (Fig. 12). The mantle fold was limited to the region of the shell gland at the posterior end of the larva.

In summary, observations on whole mounts and sections showed that arrest at the veliger stage entailed inhibition of the differentiation and proliferation of mesenchyme cells of the body and foot, and inhibition of proliferation and enlargement of the endoderm cells of the larval liver.

Treated and normal 1-, 2- and 3-day embryos. In order to determine the degree of endodermal and mesodermal differentiation prior to the appearance of exogastrulae and arrested veligers, sections were prepared of 1-, 2- and 3-day-old *L. stagnalis* embryos. The treated group had been exposed to 4×10^{-5} M $CoCl_2$ for the first 24 hours.

The lumen of the blastocoel of the normal 1-day embryo was characteristically filled with mesomeres containing darkly staining cytoplasm and many γ granules (Fig. 13). There were fewer mesomeres and a larger blastocoel in the cobalt-treated embryos (Fig. 14). Neither normal nor treated embryos had begun to gastrulate.

Treated and normal 2-day-old embryos had gastrulated (Figs. 15, 16). In the normal gastrulae the blastocoel was obliterated by the invaginated endomeres (Fig. 15). Furthermore, there was intimate contact between the endomeres at the tip of the archenteron and the overlying ectomeres, and also between endomeres, mesomeres and ectomeres, as had been previously reported (Raven, 1952a). The endoderm cells lining the archenteron contained material that stained as the capsule-fluid albumen trapped in the archenteron. In the cobalt-treated embryos the mesomeres were limited to a few cells at the vegetal end of the blastocoel, which was not filled with cells. The endomeres had invaginated and their protoplasmic processes extended toward the inner side of the ectoderm (Fig. 16); the failure of the endomeres to make contact with the ectomeres may be due to a fixation artifact. If the cobalt-treated embryo sectioned for Figure 16 had been allowed to develop, it probably would have exogastrulated or developed into an abnormal veliger.

Normal 3-day embryos had reached the late "trochophore" stage (Raven, 1946) (Figs. 7, 17); the shell gland was present and was in intimate contact with the

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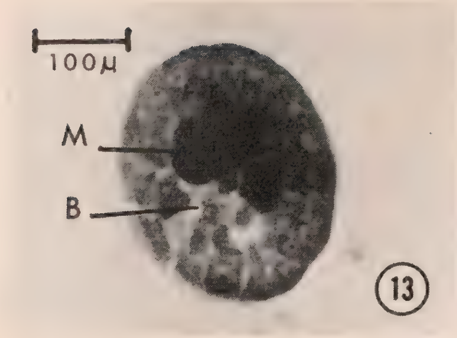
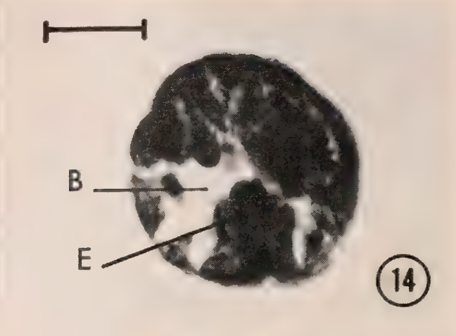
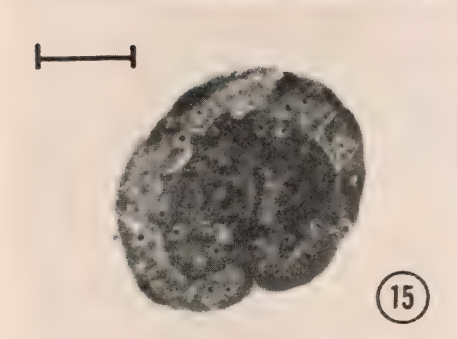
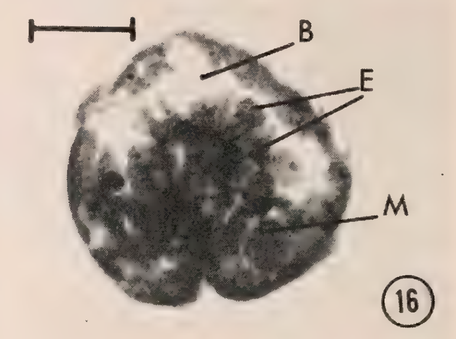
hours with 4×10^{-5} M $CoCl_2$. Body and foot collapsed during the fixation and dehydration process.

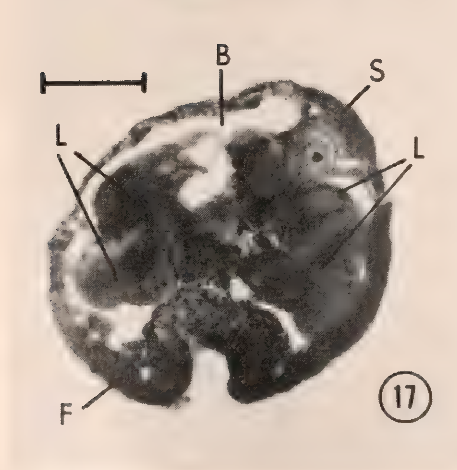
Key for Figures 7 to 12. F, foot; L, larval liver; M, foot mesenchyme; R, radula sac; S, shell gland; St, stomodeum.











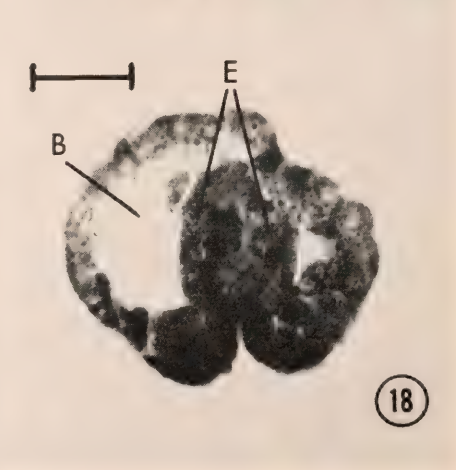


FIGURE 13. Section of a normal 1-day old embryo.

FIGURE 14. Sagittal section of an abnormal 1-day-old embryo treated the first 24 hours with 4×10^{-5} *M* $CoCl_2$.

FIGURE 15. Sagittal section of a normal 2-day-old embryo.

FIGURE 16. Sagittal section of an abnormal 2-day-old embryo treated the first 24 hours with 4×10^{-5} *M* $CoCl_2$.

FIGURE 17. Sagittal section of a normal 3-day-old embryo.

FIGURE 18. Sagittal section of an abnormal 3-day-old embryo treated the first 24 hours with 4×10^{-5} *M* $CoCl_2$.

Key for Figures 13 to 18. B, blastocoel or body cavity; E, endoderm cells; F, foot primordium; M, mesoblasts; L, larval liver cells; S, shell gland primordium.

small-celled endoderm. The stomodeum was differentiated into an oral cavity and a radula sac. Large albumen-filled larval liver cells filled most of the body cavity and extended anteriorly between the radula sac and foot *anlagen*, which consisted of a small bulge of ectoderm that had proliferated several layers of cells. Spindle-shaped mesenchyme cells were scattered in the body cavity between the larval liver cells and the body wall ectoderm. The treated 3-day embryos were smaller than the controls, lacked a stomodeum and radula sac, had few mesenchyme and few larval liver cells (Fig. 18). However, as Figure 18 shows, there was intimate contact between the tip of the archenteron and the overlying ectoderm.

Sections of 1-, 2- and 3-day-old cobalt-treated embryos support the idea that cobalt inhibits the proliferation of the mesomeres and growth of the larval liver cells. The reduction in size and number of these two types of cells results in (1) a partially cell-filled blastocoel cavity following gastrulation, and (2) a lack of intimate contact between the endodermal and mesodermal cells and the ectodermal cells. Continued ectodermal proliferation and uptake of water or other fluids by the blastocoel may cause formation of a vesicular or dumbbell-shaped exogastrula. In less extreme instances the abnormal embryo might differentiate into an abnormal veliger or snail.

24-hour treatment with other metal salts

Lallier (1955a, 1955b) and Rulon (1953, 1955, 1956, 1957) showed that chloride salts of cadmium, zinc, nickel, manganese and cobalt caused similar morphological abnormalities in echinoderm embryos. In the present study a series of experiments was run to test the morphogenetic activity of cadmium, nickel and manganese ions on *L. palustris* eggs. All three ions produced morphological abnormalities similar to the abnormalities obtained following cobaltous chloride treatment. The minimum concentrations of these several ions and of cobalt which produced the maximal direct mortality, maximal arrested veligers, and maximal normal snails are summarized in Table III. The sequence of effective concentrations is Mn < Co < Cd < Ni.

TABLE III

Effective molar concentrations of cobaltous chloride and other metal chlorides on L. palustris eggs

Salt	Maximum direct mortality	Maximum arrested veligers	Maximum normal snails
$MnCl_2$	5×10^{-5} *M*	2×10^{-5} *M*	1×10^{-5} *M*
$CoCl_2$	4×10^{-5} *M*	1×10^{-5} *M*	2×10^{-6} *M*
$CdCl_2$	6×10^{-6} *M*	2×10^{-6} *M*	1×10^{-6} *M*
$NiCl_2$	8×10^{-6} *M*	4×10^{-6} *M*	2×10^{-7} *M*

DISCUSSION

Cobaltous chloride causes several characteristic malformations in *Limnaea*; the cobalt-sensitive elements appear to be the fourth quartette of micromeres and their derivatives, the mesomeres and larval liver cells. A variety of agents including

Ni^{++}, Cd^{++}, Mn^{++}, alkali metals, alkaline earth metals, centrifugation, and heat shock produce similar abnormalities (Raven, 1942, 1952a, 1956; Raven and Dudok de Wit, 1949; Raven and Kovoets, 1952; Raven and van Egmond, 1951; Raven and van Erkel, 1955; Raven and Spronk, 1952; Raven *et al.*, 1947; Geilenkirchen, 1961; Geilenkirchen and Nijenhuis, 1959). Exogastrulae, arrested veligers, and hydropia also occur in eggs laid and developed in aquaria, and are common in eggs collected in natural habitats.

Exogastrulae and hydropia

Exogastrulation in *Limnaea* has been ascribed to nonspecific action on the material of the vegetal hemisphere (Raven, 1952a, 1952b), to injury of the cortical factors involved in ooplasmic segregation (Raven, 1958) and to interference with the differentiation and invagination of the endomeres, causing accumulation of fluid in the blastocoel (Geilenkirchen and Nijenhuis, 1959). The latter authors also suggest that impairment of osmoregulatory mechanisms, such as the larval kidneys, may partially be responsible. The observations presented in this paper suggest that inhibition of proliferation and differentiation of the mesomeres may be the cause of exogastrulae and hydropia.

Raven (1946, 1958) reported that gastrulation in *Limnaea* occurs by invagination of the endomeres. By means of pseudopodia the endomeres connect with the inner side of the cells of the animal hemisphere and with the mesomeres occupying the ventral, posterior and lateral regions of the blastocoel (Fig. 15; Raven, 1946). Cobalt treatment appears to suppress the formation of the primary mesoblasts (Figs. 14, 18). As a result there are relatively few mesomeres present in the blastocoel at the time of endomere invagination; the surfaces of the mesomeres may be altered, reducing also the affinity and adhesion between the mesomeres and ectomeres. Nevertheless, the endomeres sometimes do invaginate (Figs. 16, 18), but their pseudopodia do not always effect contact with the ectomeres across the abnormally large blastocoel (Fig. 16). Despite the lack of mesomeres, invagination may be completed if contact between the endomeres and ectomeres is established (Fig. 18). During this process, the blastocoel swells, probably because colloidal material accumulated in the blastocoelic fluid causes increased water uptake. When the gastrula hatches from the vitelline membrane, the fluid-filled space may enlarge further, producing a hydropic vesicular exogastrula.

Inhibition of proliferation and differentiation of the mesomeres may also be responsible for the hydropic foot and body cavity of larvae and shell-less snails. Some arrested larvae appeared to have more mesenchyme than others, particularly in the foot region; the degree of inhibition of the primary mesomeres and the extent of proliferation of mesenchyme cells from the epidermis of the foot and body may cause variation. An additional contributing cause may be impairment of the larval kidneys, which appear, at least in *Physa*, to be derivatives of the two primary mesomeres (Wierzejski, 1905).

Similarly, heavy metal ions cause exogastrulation and hydropic larvae in echinoderms, partly by inhibiting the mesenchyme-forming regions. Hydropic larvae with isolated mesenchyme cells and abnormal spicules occur (Mateyko, 1961; Lallier, 1956; Rulon, 1955). Radial larvae and polar-elongated larvae, described by Rulon (1955) and Lallier (1956), may also result from abnormal differentiation of the mesenchyme cells.

Arrested larvae

The normal *Limnaea* embryo incorporates capsule fluid by pinocytosis as early as the 40-cell stage (Raven, 1946; Elbers and Bluemink, 1960); ectodermal incorporation of capsule fluid continues after gastrulation and is progressively localized in the velar cells and large cells of the head vesicle (Raven, 1946). During the third day of development the esophageal process of the stomodeum coalesces with the mid-gut and the capsule fluid is actively ingested *via* the digestive tract. The ectoderm cells no longer stain for capsule fluid and the larval liver cells become the main sites of capsule fluid digestion (Raven, 1946, 1958; Bloch, 1938). Apparently cobalt elicits arrested larvae and shell-less snails by inhibition of liver cells. The resultant inadequate utilization of the capsule fluid eventually starves these larvae. Although the small cobalt-treated larvae had only a few, irregularly stained liver cells, they lived for several days. Evidently their larval liver was able to sustain minimal metabolism but not support growth. Variation in the functional capacity of the larval livers may have caused the observed differences in development of arrested veligers.

Mechanism of action of cobalt

Our current ignorance of the structure and function of cells and of the nature of embryonic development makes interpretation of the cobalt effect difficult. The biological effect of metal ions is undoubtedly due either to their combination with free anionic groups of functional significance or to replacement of biologically critical cations. In the case of *Limnaea*, the site of action must lie in the fourth quartette of micromeres and persist in their derivatives, the mesomeres and larval liver cells.

Non-uniform distribution of functional groups has been shown in *Limnaea* eggs and other organisms. Raven (1946) found non-uniform distribution of bound —SH groups in the gastrula and trochophore stages of *Limnaea*. Fauré-Fremiet and Mugard (1948) observed in *Teredo* eggs cortical localization of an argyrophilic substance, perhaps lipoprotein in nature, which appeared to be related to detergent-sensitivity of these eggs. Differentiating echinoderm eggs showed progressive segregation of reactive groups (Immers, 1961, 1962; Bäckström, 1957, 1961). Our results support the idea that there is a cobalt-sensitive macromolecule, located in the vegetal region of the *Limnaea* egg, which is progressively segregated into the large-celled endomeres and mesomeres.

Until further studies are made, the nature of the macro-molecule can only be hypothetical. Immers' studies (1961, 1962) suggest sulfated polysaccharides as likely sites of metal-reactivity. Cobalt is known to mask —SH groups of free and protein-bound cysteine (Summer and Somers, 1953; Kinoshita, 1955; Gurd and Wilcox, 1956) and to complex with amino groups of free and bound basic amino acids (Lehninger, 1950; Williams, 1953). It is equally possible that cobalt acts by displacement of critical cations; Raven (1956) and Geilenkirchen (1961) have suggested that the biological effects of high concentrations of Li^+, Na^+ and K^+ on *Limnaea* may be due to displacement of calcium.

It is easy to postulate the existence of cobalt-reactive groups in molecules critical to development; the problem of the future is to identify such receptor sites and to understand their role in normal development.

I wish to express my gratitude to the following: Professor Chr. P. Raven for several thoughtful discussions, H. Van Kooten for the photomicrographs of Figures 1 to 6, and H. H. Baldwin for aid with the manuscript.

SUMMARY

1. The effect of cobaltous chloride ($CoCl_2$) on the developmental stages of *Limnaea stagnalis* and *L. palustris* has been studied.

2. Eggs and embryos were treated for 24 hours with various concentrations of $CoCl_2$. Effective concentrations of $CoCl_2$ ranged from 1×10^{-5} to 1×10^{-4} M for *L. stagnalis* and from 2×10^{-6} to 8×10^{-5} M for *L. palustris*.

3. Variations in resistance to $CoCl_2$ were observed between species at the same stage of development, between eggs treated at different stages of development and between eggs from the same egg masses treated at the same stage of development.

4. Treatment with $CoCl_2$ resulted in (a) separation of blastomeres, (b) vesicular and dumbbell-shaped exogastrulae, (c) arrested gastrulae, (d) veligers with a reduced larval liver, shell and foot, and with a fluid-filled body cavity containing few mesenchyme cells, (e) shell-less snails with a reduced larval liver and shell, and (f) helmet-shelled snails with abnormally wide shell apertures.

5. Manganese, cadmium and nickel produced malformations similar to those caused by cobalt. The sequence of effective concentrations was $Mn < Co < Cd < Ni$.

6. Sections of normal and cobalt-treated embryos showed an inhibition of the differentiation and proliferation of mesenchyme cells of the body and foot and an inhibition of proliferation and enlargement of the endoderm cells of the larval liver.

7. Exogastrulae and hydropic exogastrulae and veliger larvae appear to be caused by an impairment in the development of the mesomeres plus a concomitant uptake of water by the blastocoel or body cavity.

8. Arrested veliger larvae and the differences in their development are explained on the basis of variation in the functional capacity of their larval livers.

9. The morphological evidence indicates that the several characteristic malformations result from an inhibition of the cobalt-sensitive fourth quartette of micromeres and their derivatives, the mesomeres and larval liver cells.

10. The results suggest that metal ion-reactive groups in the vegetal region of the *Limnaea* egg may be progressively segregated into the fourth quartette of micromeres.

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COMPARATIVE STUDIES OF THE OXYGEN CONSUMPTION OF THREE SPECIES OF NEOTENIC SALAMANDERS AS INFLUENCED BY TEMPERATURE, BODY SIZE, AND OXYGEN TENSION[1]

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As a result of the mechanism of natural selection, populations of organisms have arisen that exhibit different degrees of physiological adaptation. Numerous facets of this subject have been discussed extensively in review papers by Prosser (1955) and by Bullock (1955).

Vernberg (1959a, 1959b) has recently investigated physiological variations in latitudinally isolated populations of fiddler crabs. As a result of these studies he has suggested that the metabolic response of the organisms has real significance as to their distribution, and points out that in the course of evolution the various populations that he studied appeared to be metabolically adjusted to the temperature fluctuation of their habitat. This problem of variation in several different marine animals, collected at different latitudes, has also been studied by Fox and Wingfield (1937), and many others.

Bullock (1955) makes clear that it is not necessary for wide geographical gaps to exist between organisms in order for them to show significant physiological variations, which in turn may affect their distribution. Illustrative of this point is the recent study of Wiens and Armitage (1961) conducted on two species of crayfish (one of which is an inhabitant of permanent ponds and running streams, and the other of temporary roadside ditches) that exist in the same area. The two species are rarely found in association with one another.

At the present time there is keen interest in the value of physiological studies in providing a greater depth of understanding of some of the problems of evolution, ecological distribution, and speciation. Prosser (1955) has called attention to the fact that (p. 229) "comparison of physiological adaptations should contribute much to an understanding of interspecific relations, intraspecific variation, and the bases for ecological ranges."

The literature on salamander respiration is not extensive. Vernberg (1952; see this paper also for earlier references) measured oxygen consumption of two species of salamanders at different seasons of the year at two different temperatures. He found seasonal differences to occur, although a correlation with habitat temperature was not clear. Species differences were noted. Much earlier, Evans (1939) had examined various factors influencing the oxygen consumption of

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[1] Supported by state-appropriated funds for organized research.

[2] This report was prepared at the Marine Biological Laboratory, Woods Hole, Mass., during the summer of 1963 while the senior author was a National Science Foundation, Science Faculty Fellow.

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several species of plethodontid salamanders. He was able to correlate activity of a species with habitat and oxygen consumption, and in some cases he noted that oxygen consumption was dependent on oxygen tension. Several investigations of the metabolic rate of salamanders during embryogenesis have been reported (Løvtrop and Werdinius, 1957; for review of this literature see Boell, 1955).

The Balcones escarpment region of the Edwards plateau of south central Texas is occupied by several forms of neotenic salamanders, five of which belong to the genus *Eurycea* (Baker, 1961). Three species of this genus are restricted in distribution to certain specific areas along the escarpment. The possibility exists that the different environmental conditions required by these three forms may be related to their individual metabolic requirements. This report deals with a study of oxygen consumption of these salamanders as influenced by temperature, body size, and oxygen tension; and was originally undertaken with the idea that the data obtained might correlate with the restricted distribution of the organisms.

METHODS AND MATERIALS

The three species of salamanders used in this investigation were collected from their native habitat (Table III) and were transported to the laboratory in glass aquaria filled with water and aquatic vegetation collected with the animals. The specimens were maintained in an air conditioned laboratory (23 ± 2° C.) until respiratory measurements were made. The salamanders were not fed (other than the food material present in the water and vegetation collected with them), and were never kept in the laboratory for longer than a week. Respiratory measurements were frequently made on the same animal more than once.

All oxygen consumption measurements were made with the Warburg constant volume respirometer. The direct method was employed, as described by Umbreit *et al.* (1957). In addition to KOH as a CO_2 absorbent in the center well, each reaction vessel contained three milliliters of water taken directly from the aquaria in which the salamanders were maintained. Control experiments established the fact that neither pH nor nitrogen content of the water changed significantly during the course of a measurement. Following the respiratory determinations the specimens were removed from the reaction vessels, placed in small plastic containers, and weighed to the nearest milligram. For salamanders over about 250 milligrams only one animal constituted a sample; however, for the smaller specimens more than one was placed in each reaction vessel. Generally an experimental sample consisted of one large, two medium, or three small animals per reaction vessel.

Respiratory measurements were made both when the manometers and reaction vessels were not shaken, and when they were shaken in the usual manner (135 oscillations per minute through a 5-cm. excursion). In the results this is termed with, and without, mechanical stimulation. When no shaking was done, particular care was taken to keep the rate of gas exchange low (by using small samples) in order to insure that the rate of oxygen diffusion from the gas phase to the liquid phase did not become a limiting factor. Indeed, that this was accomplished, and that diffusion did not become limiting may be inferred from both the shape and the slope of the curves shown in Figures 1, 2, and 3.

Following an equilibration period of 15 to 20 minutes, manometer readings were taken at 15-minute intervals for a period of two hours. To minimize variations between individual readings, these raw data were plotted and the hourly rate determined from the slope of the resulting line. Oxygen consumption is expressed in cubic millimeters per gram (wet weight) per hour.

The following two items should be mentioned: (1) no efforts were made to acclimate the salamanders to the temperature of the measurements, other than the equilibration period described above, and (2) rhythms were not taken into account, except that approximately one-half the measurements were made in the morning and one-half in the afternoon. Ralph (1957) has observed a diurnal rhythm of locomotor activity in *Plethodon cinereus* that is modified by an influence having a lunar frequency.

RESULTS

Influence of temperature and mechanical stimulation on oxygen consumption

There is recorded in Table I a summary of the mean rate of oxygen consumption (mm.^{3}/gm./hr.), for each temperature and body size, of the three species

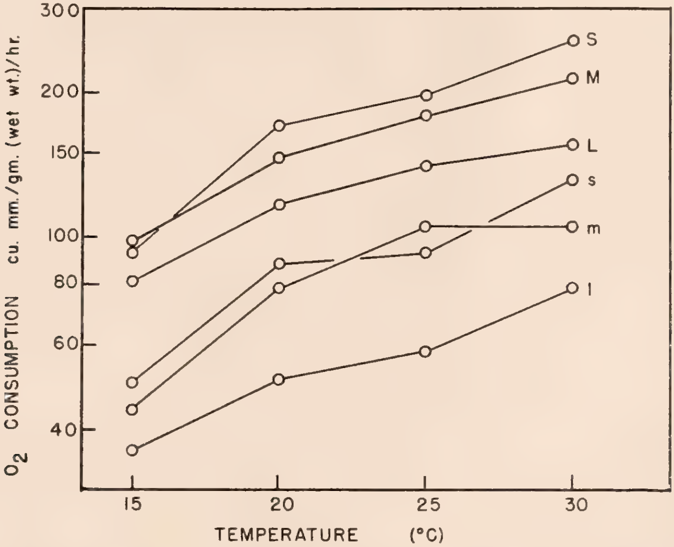

FIGURE 1. *Eurycea neotenes:* Relation of oxygen consumption to temperature and body size, plotted semi-logarithmically. S, M, L (small, medium, and large body size—mechanically stimulated). s, m, l (small, medium, and large body size—without mechanical stimulation).

of salamanders studied, both with and without mechanical stimulation. The number of measurements and standard deviations are shown. Figures 1, 2, and 3 present these data in graphic form. It is evident that *Eurycea neotenes* (Fig. 1) shows an increased rate of oxygen consumption at each higher temperature. The most rapid increase occurs between 15 and 20° C., while the rate of increase between 20 and 30° C. is somewhat less but appears to be more regular. Figure 2 for *Eurycea nana* reflects a fairly regular increase in the rate of oxygen consumption from 15° to a maximum at 25° C.; however, the curves reverse at 25°C. and

TABLE I

Mean oxygen consumption rate for three species of salamanders
(mm.^{3}/gm./hr.)

Animal size (Range in gm.)	Temperature (° C.)							
	15		20		25		30	
	Still	Shaken	Still	Shaken	Still	Shaken	Still	Shaken
				Eurycea nana				
Small (0.01–0.1)	5/58±14.5*	5/89 ±10	5/77 ±16.1	6/131±51.2	10/106±29.7	24/199±37.9	—	8/183±30.8
Med. (0.1–0.208)	6/48± 9.5	5/68 ±15.8	3/71 ±18.1	5/117±26	8/86 ±25	22/166±31.8	—	8/158±26.3
Large (0.216–0.431)	5/40± 4.9	5/49 ± 2.8	9/55 ±16.9	9/92 ±20.2	15/81 ±18.1	19/155±20.6	—	6/144±25.9
				Eurycea neotenes				
Small (0.101–0.148)	6/50± 9.5	4/93 ± 7.8	8/88 ±26.3	7/170±16	5/93 ± 8	4/197± 9	3/132± 7.1	5/253±45.7
Med. (0.159–0.327)	4/44± 8.1	4/98 ±21.3	4/79 ±18.1	6/146±23.2	4/106± 7.9	3/179±31.8	4/105± 6.2	5/214±24.6
Large (0.756–0.849)	6/36±10	5/81 ± 9.7	6/51 ±10.9	5/117±21	3/58 ± 3.1	4/141± 4.2	4/79±12.9	5/157±12.9
				Eurycea pterophila				
Small (0.034–0.090)	3/78± 7.8	4/142±12	4/134±31.6	7/225±28.1	4/97 ± 4.4	4/202±15.4	4/91 ±19.7	4/182±13.7
Med. (0.111–0.280)	4/84±24.3	5/134±21.5	6/116±33.3	5/179±25.9	5/90±15.6	4/161±19.3	4/82 ±15.7	4/156±23.3
Large (0.280–0.592)	4/66±21.1	5/109±24.7	4/85 ±16.6	4/147±15.9	3/65 ± 8.1	4/145±22.6	3/62 ± 4.9	4/137±22.6

* The number of measurements precedes the slant. Standard deviations are shown.

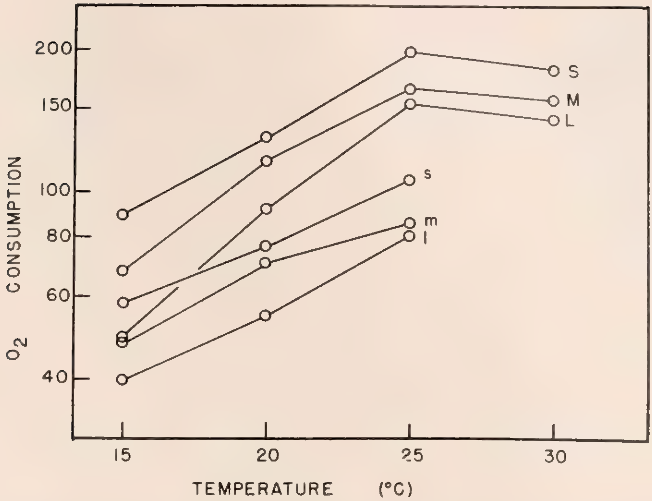

FIGURE 2. *Eurycea nana:* Relation of oxygen consumption to temperature and body size, plotted semi-logarithmically. S, M, L (small, medium, and large body size—mechanically stimulated). s, m, l (small, medium, and large body size—without mechanical stimulation).

a decreasing rate is shown between 25° and 30° C. The results obtained with *Eurycea pterophila* are revealed in Figure 3. Following a rapid increase in rate of oxygen consumption from 15° to a maximum at 20° C., the curves reverse their slope and show a decreasing rate from 20 to 30° C.

It is striking that mechanical stimulation does not alter the shape of the curves but merely shifts them up to the rate axis, *i.e.*, the same conclusions are apparent from either set of curves: those yielded when the organisms were mechanically stimulated by shaking, or those obtained when the reaction vessels were motionless.

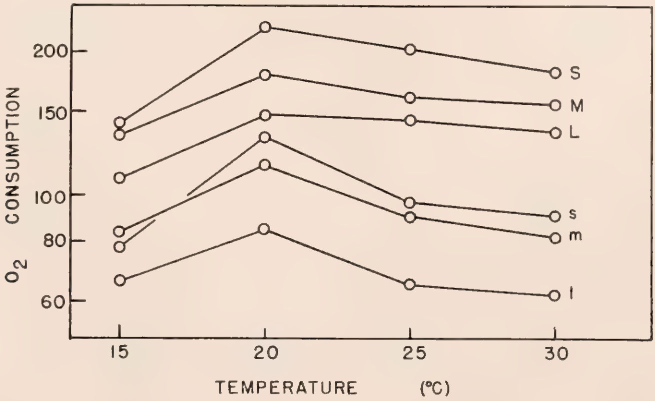

FIGURE 3. *Eurycea pterophila:* Relation of oxygen consumption to temperature and body size, plotted semi-logarithmically. S, M, L (small, medium, and large body size—mechanically stimulated). s, m, l (small, medium, and large body size—without mechanical stimulation).

Relation of body size to oxygen consumption

These data are amenable to some analysis of the effect of body size on the rate of oxygen consumption. In Table I and Figures 1, 2, and 3, the data were divided arbitrarily into three groups (small, medium, and large) on the basis of body size. It is obvious that the highest rate of oxygen consumption is shown by the smaller organisms, while the lowest rate is exhibited by the largest salamanders, and an intermediate rate is shown by medium-sized specimens. Again it is apparent that this may be observed from either set of curves, *i.e.,* the ones obtained with mechanical stimulation, or those resulting without shaking.

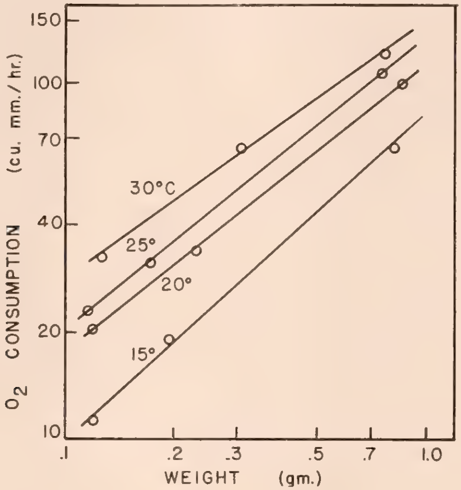

FIGURE 4. Plot of oxygen consumption *vs.* body weight for mechanically stimulated *Eurycea neotenes* at 15, 20, 25, and 30° C. (log-log plot).

Utilizing the equation given by Prosser and Brown (1961) for the relation of total metabolism to body size,

$$M = KW^b$$
$$\log M = b \log W + \log K$$

where M = total metabolism, or O_2 consumed per hour
W = body weight
b is given by the slope of the plot of log O_2 consumption against log weight
K is obtained from the Y intercept,

a further treatment of the data is possible. Figure 4 contains a plot, for mechanically stimulated *Eurycea neotenes,* of the logarithm of oxygen consumed per hour against the logarithm of the weight of the organism. Typical progression curves are yielded. The approximate values of "b" at various temperatures, as calculated from the slope of the lines, not only for *Eurycea neotenes,* but also for the other two species of salamanders studied, are presented in Table II.

TABLE II

Values of "b" for three species of salamanders at different temperatures

Temperature	*Eurycea nana*	*Eurycea neotenes*	*Eurycea pterophila*
15° C.	.68	.92	.83
20° C.	.80	.81	.75
25° C.	.79	.82	.80
30° C.	.85	.73	.80

Effect of oxygen tension on the rate of oxygen consumption

Prosser (1955) mentions that oxygen availability has not been thoroughly investigated as a factor limiting the range of species or isolating populations. With this in mind the following preliminary experiments were undertaken. Various mixtures of oxygen and nitrogen were prepared as described by Umbreit *et al.* (1957), and after the salamanders were placed in the reaction vessels and attached

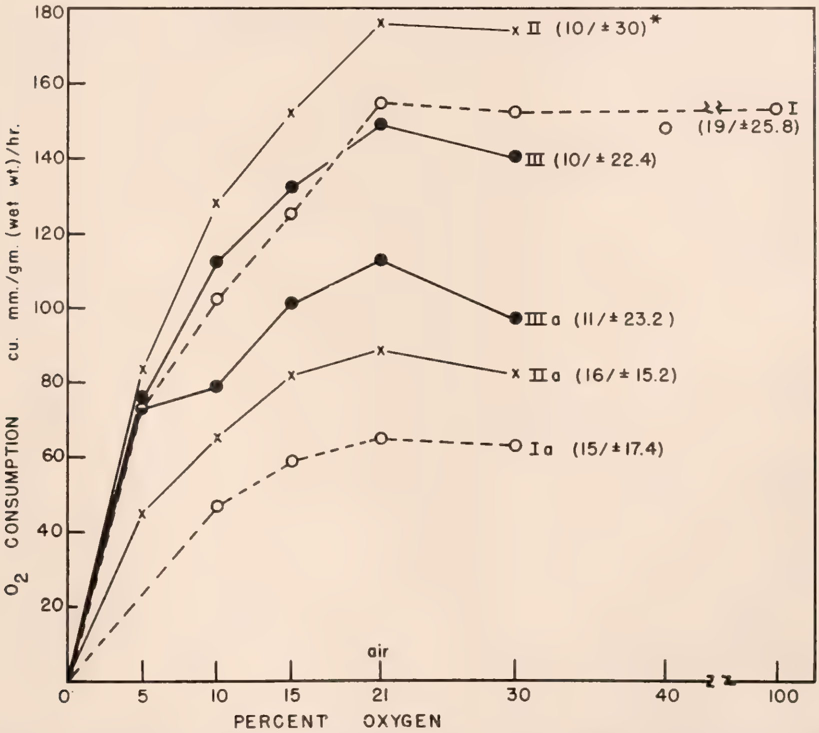

FIGURE 5. Influence of oxygen concentration on rate of oxygen consumption by mechanically stimulated salamanders at two different temperatures. I (dotted line, open circles), *Eurycea nana;* II (crosses), *Eurycea neotenes;* III (closed circles) *Eurycea pterophila.* I, II, III at 30° C.; Ia, IIa, IIIa at 15° C. *Numbers in parentheses indicate number of measurements and standard deviation of the point plotted for air. All other points are the average of 2 to 5 measurements.

to the manometers, the gas atmosphere was changed by flushing each vessel with at last one liter of the desired gas mixture.

Curves relating the rate of oxygen consumption to the per cent oxygen in the gas mixture are presented in Figure 5. Although the curves are not as smooth as might be desired (possibly due to a relatively small number of measurements averaged for each point, other than air), it is quite clear that the usual type of hyperbolic curves result. The maximum rate of oxygen consumption occurs in air in each case. Below the oxygen content of air the rate of oxygen consumption is related to oxygen availability. Just why a slightly decreased rate of oxygen consumption is shown in an atmosphere of 30% oxygen is puzzling; however, due to the limited number of determinations made under these conditions, as well as the standard deviation exhibited by the points plotted for air (see Fig. 5), this decrease is not considered significant.

DISCUSSION

The fact that the highest rate of oxygen consumption shown by the salamanders investigated occurs at a different temperature for each species is most interesting. Specifically, the maximal rate for *Eurycea neotenes* occurs at 30° C., for *Eurycea nana* at 25° C. and for *Eurcycea pterophila* at 20° C. Mechanical stimulation (shaking) causes approximately a two-fold increase in the rate of oxygen consumption (Figs. 1, 2, 3). Mechanical stimulation does not change the shape of the R/T curves, but only relocates them higher on the rate axis.

The correlation of these results with natural habitat conditions of the salamanders may be of importance. There is briefly summarized in Table III information pertaining to the habitat. In nature *Eurycea nana* is subjected to the least variable temperature and the lowest oxygen content. *E. nana* shows maximal oxygen consumption at 25° C., which is slightly above the habitat temperature, but oxygen uptake is decreased at a temperature of 30° C. With regard to *E. nana* a qualitative observation was made which seems pertinent. In measuring oxygen consumption (no mechanical stimulation), it was noted that about one-half the organisms died when subjected to a temperature of 30° C. for a little over two hours. Of course in these cases no data were obtained. At 25° C. about one out of every seven animals died. Mechanical stimulation lessened the mortality, as only about one out of ten perished at 30° C. and none died at 25° C. For the other two species of salamanders no lethal effects due to the higher temperatures were observed. This seems particularly unique in the case of *E. pterophila* which exhibits a maximal respiratory rate at 20° C. These observations may mean that *E. nana* is the least tolerant of temperature change, which is in accord with the almost constant temperature of its natural habitat.

Just why *E. pterophila* should show a maximal metabolic rate at 20° C., with a decreased oxygen consumption both at 25° and 30° C., while *E. nana* shows a maximal rate at 25° C., and a decreased rate at 30° C., is not clear, particularly when the temperature of the natural habitat is essentially the same in both cases. Possibly the explanation lies in different characteristics of the enzymatic machinery of the two species. With regard to enzyme activity it is clear that as temperature is increased two forces come into play: the rate of destruction of the enzyme, versus the increase in rate of substrate transformation; and at elevated temperatures, the first may overshadow the second.

The influence of temperature on the metabolism of *Eurycea neotenes* is straightforward in that oxygen consumption shows a fairly regular increase with each increase in temperature throughout the range investigated. This correlates nicely with the wider temperature variation that *E. neotenes* encounters in nature (Table III).

As pointed out by Prosser and Brown (1961), interpretation of the size correlations of metabolism are difficult. In general, small animals exhibit a higher standard metabolic rate than related animals of greater body weight (Zeuthen, 1953; Davison, 1955). The salamanders included in this study represent no exception to this rule, as may be seen from the data presented in Figures 1 through 4, as well as in Table II. Plotting the metabolism of species against body size on double logarithmic paper, Zeuthen (1953) has observed a continuous three-phase curve. For unicellular organisms the slope "*b*" of the curve is 0.7; for small metazoa, *b* is about 0.95; and for larger poikilothermic animals and for homeothermic animals *b* is approximately 0.75. Prosser and Brown (1961) state that *b* values vary from 0.55 to 1.0. Among the causes of this variation they mention body surface in homeotherms and in unicellar organisms, growth patterns, types of external respiration, increase of enzymes related to body mass, and disproportionate increase of different tissues. From Table II, *b* values for the various salamanders range between 0.68 and 0.92, with most of the values

Table III

Habitats of the three species of salamanders studied

Species	*Eurycea nana*	*Eurycea pterophila*	*Eurycea neotenes*
Locality	Spring Lake. San Marcos, Tex.	Fern Bank Springs, near the Blanco River. Eight miles west of San Marcos, Texas	Prof. Green's Place. Near Devil's Backbone. Fifteen miles southwest of San Marcos, Texas.
Description	Spring-fed, clear deep water. Abundant aquatic vegetation.	Natural springs. Clear, shallow, moderately fast flowing water. Abundant aquatic vegetation.	A spring-fed, creek or stream type habitat. Slower moving water. Somewhat less aquatic vegetation.
Temperature (Throughout a six-month period, Feb. to August.)	21–22° C.	21–22.5° C.	19–23.5° C.
Oxygen content*	3.2 ml./l.	5.7 ml./l.	4.9 ml./l.

* The figures reported are average values determined by Gary D. Henry (unpublished data). Analyses for oxygen were carried out at various times of the day over a period of several months, employing the apparatus and method described by Scholander *et al.* (1955).

falling between 0.73 and 0.85. Thus, there is good agreement between the values obtained in this study and those previously reported in the literature.

A correlation between the oxygen consumption of aquatic animals and their ecology has been frequently demonstrated. Usually, active species and inhabitants of rapid streams exhibit a higher rate of oxygen consumption than do more sluggish species and inhabitants of slow streams or standing water (Prosser *et al.*, 1950, Table 42). Often the inhabitants of oxygen-deficient water are able to maintain a steady rate of oxygen consumption when oxygen tension of the medium is falling, until a low critical level of oxygen is reached (Mann, 1956). Evans (1939) demonstrated many years ago that in some species of salamanders oxygen consumption was dependent on oxygen tension. There do not appear to be significant differences in the maximal rate of oxygen consumption exhibited by the three species of salamanders studied here, although the maximal rate does occur at different temperatures for the different species. Apparently, differences in habitat conditions (Table III) are not large enough to be reflected in the organisms' response to different concentrations of oxygen (Fig. 5). It is evident from the data of Figure 5 that below the oxygen content of air the rate of oxygen consumption is dependent on the oxygen concentration; however, this relationship is not strictly linear and thus these organisms are not "oxygen conformers" in the true sense of Prosser and Brown's (1961) definition. Neither are they very good "regulators," but probably they belong in that intermediate group of organisms between conformers and regulators.

SUMMARY

1. Three species of neotenic salamanders of the genus *Eurycea* were collected from their specific, though not widely separated, habitats on the escarpment region of the Edwards plateau in south central Texas.

2. Utilizing standard Warburg manometry, oxygen consumption was determined for each of the species (with and without shaking) at 15, 20, 25, and 30° C. Maximal respiratory rates were exhibited by *Eurycea neotenes* at 30° C., by *Eurycea nana* at 25° C., and by *Eurycea pterophila* at 20° C. Mechanical stimulation (shaking) increased the rate of oxygen uptake but did not alter the shape of the R/T curves. For *E. nana* and *E. neotenes* the temperature at which the maximal metabolic rates were observed appeared to be correlated with the temperature characteristics of the natural environment.

3. There is a decrease in metabolic rate with increasing body size. The specific relationship is in good agreement with previously reported values.

4. All three species of salamanders seem to be intermediate between oxygen conformers and regulators, in that the rate of oxygen consumption is dependent on oxygen concentration (up to the oxygen content of air), but not in a strictly linear manner.

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EFFECTS OF METABOLIC INHIBITORS ON PLANARIAN REGENERATION[1]

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This study describes abnormalities in regenerating planarians induced by inhibitors of carboxylation enzymes. The physiological significance of CO_2 fixation in animals has been in doubt. One way of assessing its importance in normal metabolism would be to inhibit fixation in a suitable test specimen and study the effects on some well-known physiological process in that specimen. This report is concerned with the influence of *p*-chloromercuribenzoic acid (PCMB) and avidin on regeneration of the head in the planarian, *Dugesia tigrina.*

It is known that enzymatic activity which depends on the free active sulfhydryl or thiol group ($-SH$) can be inhibited by various compounds, resulting in blockage of normal metabolic pathways. PCMB is a typical mercuric, mercaptide-forming inhibitor of sulfhydryl enzymes. One of the enzymes known to require free $-SH$ groups is the malic enzyme, which catalyzes the reversible decarboxylation of l-malate to pyruvate. Hargreaves (1954) worked with the isolated malic enzyme system of pigeon liver and found PCMB concentrations of 8.3×10^{-6} and 1×10^{-4} *M* inhibitory. Hammen and Lum (1962) showed the presence of CO_2 fixation in the planarian *Dugesia tigrina*, in which exposure to C^{14}-bicarbonate led to labeling of malate and other acids of the citric acid cycle. The main pathway was evidently *via* the malic enzyme system. Coldwater (1933) used the nitroprusside reaction on *Planaria maculata*, now known as *Dugesia tigrina*, and found a high concentration of the $-SH$ group in regenerating tissue during the period of most active cellular division. Thus, it seems logical that PCMB could inhibit or alter normal planarian regeneration.

In an organism's intermediary metabolism, two of the possible pathways of CO_2 fixation are *via* (a) the malic enzyme as indicated above, and (b) the propionyl carboxylase system which catalyzes the conversion of propionate to succinate. Halenz and Lane (1960) isolated propionyl carboxylase from bovine liver mitochondria, and showed that there was an associated enzyme-bound biotin, which is necessary for enzymatic activity. Avidin is a protein isolated from raw egg white, which has a specific biotin-binding capacity. Hammen and Lum (1962) demonstrated the utilization of propionate by the planarian, *Dugesia tigrina*, by means of C^{14}-labeling. Thus, by exposing regenerating planarians to avidin, one might expect some adverse effect on regeneration.

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Methods and Materials

The original stock of flatworms, *Dugesia tigrina*, was obtained from the Champlain Biological Service, Glen Gardner, N. J. The worms were maintained in filtered pond water cultures under laboratory conditions (22–27° C.) and were fed twice a week on raw beef liver. To maintain a healthy stock, culture bowls were cleaned and water changed after each feeding. To study regeneration, worms with an average weight of 4 mg. and length of 10–11 mm. were decapitated behind the auricles and sectioned through the pharyngeal region. The anterior section was that region between the head and the pharynx, and the posterior section was that region behind the pharynx. Each worm then yielded two pieces, each with the possibility of regenerating a head.

The water was obtained from a goldfish pond on the Adelphi College campus, which maintained a variety of plant and animal life. After collection the water was filtered and allowed to stand overnight to reach room temperature before use. As Jenkins (1961) and others have shown, the oxygen consumption of planarians declines quite slowly after the first week of starvation, suggesting a relatively stable state of metabolism. Therefore, all experiments were begun six days after feeding.

To study the normal rate of regeneration, daily observations were made, and the stages of regeneration were classified according to Henderson and Eakin

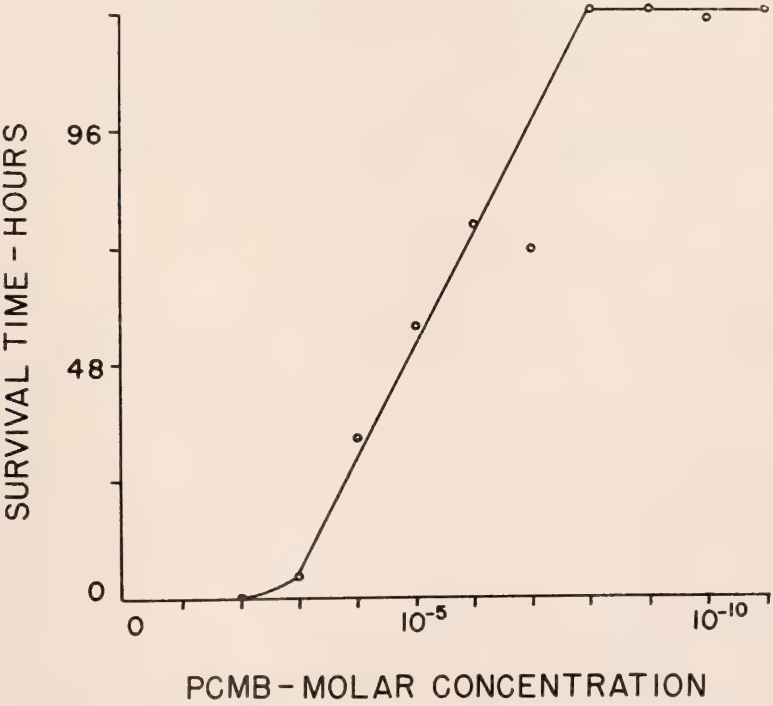

Figure 1. Survival of intact worms in varying concentrations of PCMB.

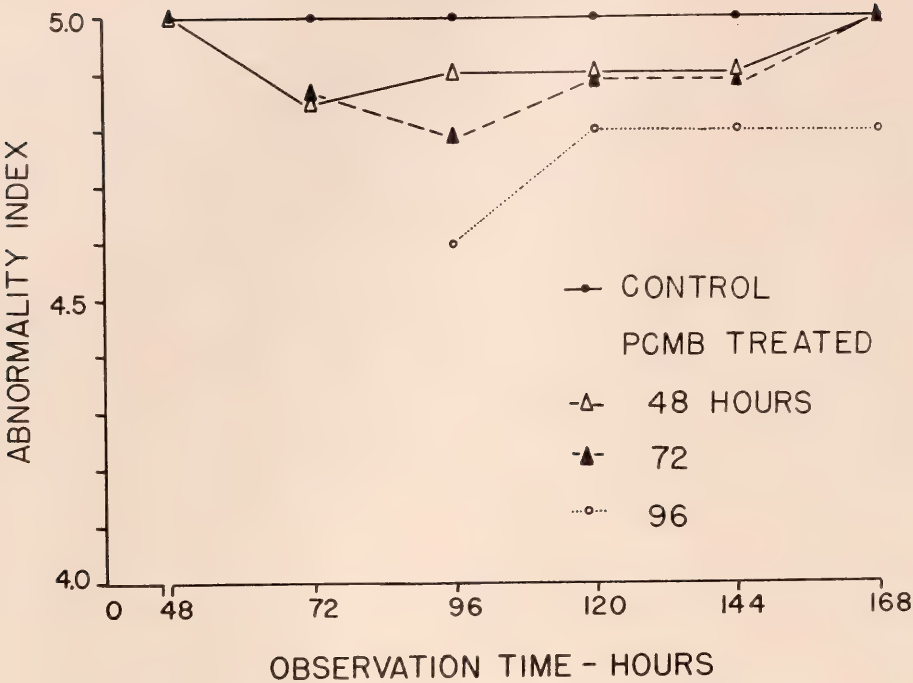

FIGURE 2. Relationship of frequency of eye abnormalities to time of exposure to 1×10^{-8} *M* PCMB.

(1959). During regeneration any abnormalities observed were classified according to Child (1911), as follows:

Index	Description
1.	Headless, no apparent outgrowth.
2.	Anophthalmic, some outgrowth but eyes absent.
3.	Teratomorphic, abnormal head shape with reduction in size.
4.	Teratophthalmic, head shape normal but eye abnormalities.
5.	Normal, appears like control in regard to head formation and eyes.

The same procedures and numbering system were applied in studying regeneration under the influence of the inhibitors.

The metabolic inhibitors used, the Na salt of *p*-chloromercuribenzoic acid (PCMB) and avidin, were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Pond water solutions of PCMB ranged from 10^{-1} *M* (37.9 mg./ml.) by 1:10 serial dilutions to 1×10^{-11} *M*. Avidin was used in concentrations of 0.4, 1, and 4 mg./ml.

To determine toxicity of PCMB, intact worms were placed in the graded series of solutions, and observed daily up to 120 hours. This time interval was chosen because the normal regeneration of sectioned worms was completed in 5 days. To study the effects of PCMB on regenerating worms, groups of 15 pieces each were placed in 1×10^{-8} M, the highest concentration at which lethal effects were not expected in 120 hours. The exposure times, chosen from preliminary experiments, were 24, 48, 72 and 96 hours after sectioning. After exposure, pieces were removed, washed, and allowed to complete regeneration in pond water. Since that experimental group which was exposed for the longest time had eye abnormalities persisting longer, the 96-hour experiment was repeated, using groups of 30 pieces each.

To study the effect of avidin on regenerating worms, they were placed in solutions of 0.4, 1 and 4 mg./ml. for 24 to 72 hours. A sample of denatured avidin was prepared by heating at 80° C. for 5 minutes. Worms were allowed to regenerate in the two test solutions of avidin and heated avidin along with pond water controls.

RESULTS

Effects of various PCMB concentrations on planarians

In preliminary experiments, the normal regeneration rate of *Dugesia tigrina* was studied in pond water, at room temperature. The anterior and posterior sections regenerated new heads at essentially the same rate, and that regeneration was complete in 5 days.

The survival time of intact worms in various concentrations of PCMB is shown in Figure 1. In concentrations of 10^{-1} and 10^{-2} *M* PCMB, death occurred in a relatively short period of time. The reactions were violent contortions, then

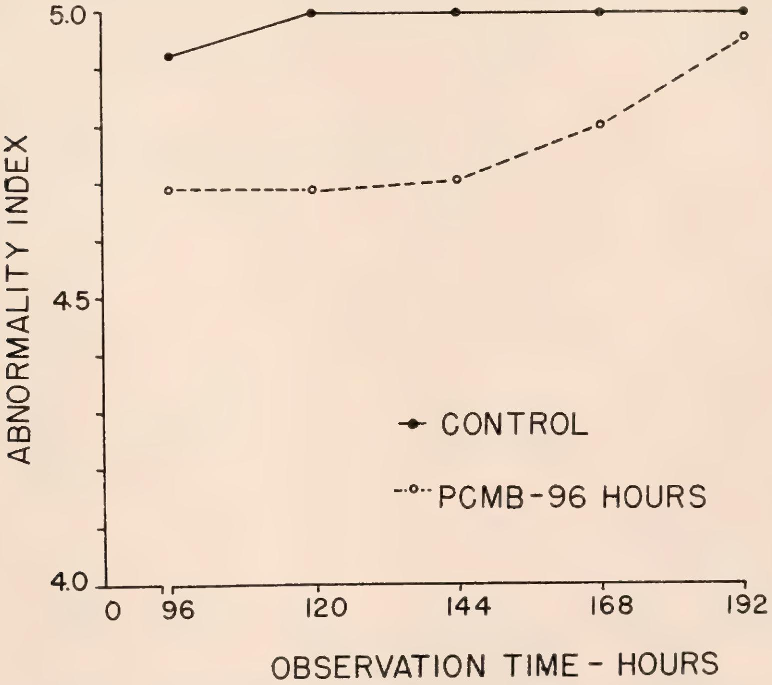

FIGURE 3. Frequency of eye abnormalities resulting from a 96-hour exposure to 1×10^{-8} *M* PCMB.

complete body paralysis, and finally cytolysis, or cellular disintegration in which the outer cellular layer peels away from the head region first and then proceeds posteriorly along the body.

In concentrations higher than 10^{-8} *M*, the worms did not survive much more than three days (77 hours), while in lower concentrations survival was long enough to observe complete regeneration. When the experiment was stopped at 121 hours, the pond water controls as well as the experimentals in concentrations lower than 10^{-8} *M* were still alive.

The rate of new head regeneration of the experimental pieces in PCMB was found to be essentially the same as in the pond water control group. The most interesting observation was an increased incidence of some form of eye abnormality in all experimental groups, relative to the small number of such defects in the controls. These were in the form of unequal eyes, eyes closer together than normal, eyes further apart than normal, one center eye, or joined eyes, the bridge of which often broke leaving two triangular eyes closer together than normal.

Degree of abnormalities related to duration of exposure

The index values of eye abnormalities resulting from different intervals of PCMB exposure during regeneration are shown by Figure 2. On observation at 168 hours, the experimental animals which had shorter intervals of PCMB exposure appeared identical to the normal controls, indicating correction of de-

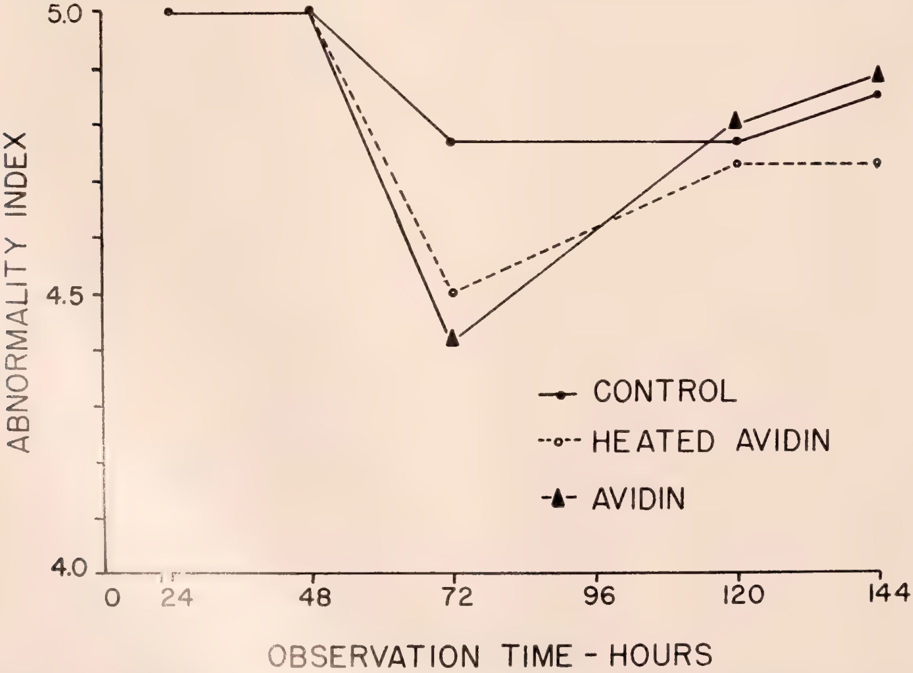

FIGURE 4. Frequency of eye abnormalities resulting from a 24-hour exposure to 1 mg./ml. of heated avidin and avidin.

fects. That experimental group which was exposed for the longest time, 96 hours, had the lowest index number, 4.6, and the most persistent eye abnormalities. A second and larger group was exposed for 96 hours and the results were as shown by Figure 3. In this group the index was first at 4.7, then rose to near 5.0 as the abnormalities tended toward self-correction.

Since it was found in a preliminary experiment that the lower concentration of avidin (0.4 mg./ml.) produced little effect and the higher (4 mg./ml.) had a lethal effect, a concentration of 1 mg./ml. was used with a 24-hour exposure. The results of this avidin experiment are shown by Figure 4. The rate of head regeneration in the test solutions was not significantly different from that of the pond water controls. Some eye abnormalities, mostly joined eyes, were found in the control group, but they tended toward self-correction. Avidin caused the greatest number of abnormalities, index 4.4, while heated avidin had a lesser effect. As in PCMB experiments, there was a tendency to correction of the defects.

Discussion

Influence of PCMB on planarian regeneration related to malic enzyme inhibition

Exposure of worm sections to PCMB solutions for various intervals during their regeneration seemed a promising approach, because Henderson and Eakin (1959) had stated that different enzyme inhibitors are effective at different stages in planarian regeneration. It was found in a preliminary experiment that if intact worms were pretreated with PCMB before sectioning, there was a higher incidence of abnormalities in both control and experimental groups. This occurrence of eye abnormalities was perhaps due to the additional deleterious effect of starvation.

On exposure to high concentrations of PCMB, the worms reacted in the manner described by Murray (1928), who classified the reactions of *Dugesia dorotocephala* to salt solutions as contortions, paralysis, and cytolysis. Hinrichs (1924), in describing the effects of caffein on the same species, stated that disintegration usually starts at the head region and proceeds posteriorly along the body, as a result of the drug having specific action on specific tissues.

Since PCMB is a sulfhydryl-binding agent, it may react with an enzyme which is –SH-dependent for activity, thus causing inactivation. Ochoa, Mehler and Kornberg (1947) isolated from pigeon liver extracts a TPN-specific enzyme which catalyzes the reversible oxidative decarboxylation of l-malate to pyruvate and CO_2 in the presence of divalent manganese ions. Ochoa *et al.* (1947) referred to this TPN-specific enzyme as the "malic enzyme." Hargreaves (1954) studied inhibition by PCMB of the activity of this enzyme, and showed that it requires free –SH groups for activity. PCMB has been reported (Hilton and Smith, 1955) to inhibit a number of sulfhydryl-containing respiratory enzymes of fungal origin, including malic dehydrogenase of the citric acid cycle, so it is clear that PCMB inhibition is not specific to the malic enzyme, but may apply to other sulfhydryl-containing enzymes in the regenerating tissue.

The results of this work show that PCMB can alter normal planarian regeneration. Worm pieces exposed to PCMB for 96 hours during their regeneration had eye abnormalities which persisted longer than those of groups which had shorter PCMB exposures. Since it is implied by other work that *Dugesia tigrina* possesses the malic enzyme system (Hammen and Lum, 1962), and PCMB is known to inhibit this enzyme, one could conclude that there is a possible relationship between CO_2 fixation and the grossly observable abnormalities in regenerating planarians.

Influence of avidin on planarian regeneration related to propionyl carboxylase inhibition

Barban and Ajl (1951) proved the reversibility of the decarboxylation of succinic acid to propionic acid and CO_2 when they demonstrated the fixation of $C^{14}O_2$, from labeled bicarbonate into succinate by *Propionibacterium pentosaceum*. Lardy and Peanasky (1953) concluded from work with extracts of rat liver mitochondria that ATP and divalent magnesium ions are necessary for the carboxylation of propionate to yield radioactive succinate.

Flavin, Ortiz and Ochoa (1955), in a study of propionate carboxylation by extracts of pig heart, found evidence of an intermediate compound, methylmalonyl-Coenzyme A (CoA), an isomer of succinyl-CoA. Tietz and Ochoa (1959) referred to the enzyme which reversibly catalyzes the conversion of propionyl-CoA to methylmalonyl-CoA as propionyl carboxylase. The enzyme requires biotin for activity, as Halenz and Lane (1960) demonstrated with their avidin studies. Avidin caused inhibition of the propionyl carboxylase enzyme system, leading to a decreased amount of $C^{14}O_2$ fixation with increasing concentrations of avidin. Since avidin pretreated with biotin did not cause inhibition, it can be concluded that the influence of avidin on propionyl carboxylase is biotin-specific. Biotin is also required for the activity of phosphoenolpyruvate carboxylase, which catalyzes the fixation of CO_2 into oxaloacetic acid. In fact, biotin is not known to serve any other function than cofactor in carboxylation reactions.

In this investigation, avidin altered normal planarian regeneration. Worm sections exposed to either avidin or heated avidin formed abnormal eye spots, avidin having a greater initial effect than heated avidin. The reason for an effect of the denatured compound is unknown. Since it has been shown that the planarian utilizes propionate (Hammen and Lum, 1962) and that avidin inhibits the biotin-requiring propionyl carboxylase, the results of the avidin experiments may be interpreted as additional evidence of a possible relationship between CO_2 fixation and grossly observable abnormalities in regenerating planarians.

The work reported is taken from the thesis of Agnes A. Smith, presented in partial fulfillment of the requirements for the Master of Science in the Department of Biology at Adelphi College, Garden City, L. I., N. Y.

SUMMARY

1. In this investigation, treatment of regenerating worms with 10^{-8} M *p*-chloromercuribenzoic acid (PCMB) and 1 mg./ml. avidin resulted in the development of defective eye spots, which were scored according to a numbering system based on Child's classification.

2. The rate of regeneration was not significantly altered by treatment with PCMB and avidin in the concentrations used.

3. A direct relationship was found between duration of exposure to PCMB solutions and frequency of abnormalities.

4. These results indicate that inhibitors of carboxylation enzymes are effective in producing defects in planarian regeneration.

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SPECIFIC INHIBITION OF REGENERATION IN CLYMENELLA TORQUATA[1]

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The notion that the extent to which an organ or tissue differentiates and grows may be limited by factors produced within the tissues themselves or by other tissues is relatively old. Hans Spemann first mentioned it in 1904, in discussing the results of his experiments on lens formation in *Triton*. E. I. Werber again stated the hypothesis in 1918, in discussing the phenomenon of lens regeneration in the salamander (p. 247): "According to our assumption it might perhaps be imagined that every tissue (or structure) elaborates during its development a substance (an 'antibody') which inhibits its growth beyond certain limits." H. W. Rand (1924) came to the same conclusion, apparently independently, in studying the regeneration of *Hydra* and *Planaria*. However, this hypothesis and its interesting implications became submerged in the wake of the exciting search for specific inductors which was stimulated by the brilliant experiments of Hans Spemann and his students.

C. M. Child, however (*e.g.*, 1929), maintained throughout the years that dominance and subordination are important forces acting in the development of the patterned individual. The hypothesis lay forgotten until 1952, when it was again restated by S. M. Rose and by Werner Braun, working independently on widely differing organisms. Rose worked on *Tubularia* and tadpoles, while Braun investigated population changes in bacteria. The next year, Gustafson (1953) arrived at the same hypothesis, also independently, while studying sea urchin development.

Since that time, much evidence has accumulated to support the dominance-inhibition theory of regeneration and differentiation, and the reader may consult Rose (1957) for a comprehensive review.

The present investigations have been undertaken in an effort to extend the validity of this theory to the annelids, the organism chosen in this case being the 22-segment polychaete, *Clymenella torquata*. This worm has been chosen for three reasons: (1) Sayles (1942) showed that the anterior and posterior ends of the worm possess differing capacities to respond to a regenerative stimulus; (2) *Clymenella* is an intertidal form, living in sandy mud in the Woods Hole region. Not only is it plentiful, but being an intertidal form, it possesses great capacity to withstand wide variations in temperature, stagnant water, and low oxygen tensions. It is thus an ideal laboratory animal. (3) In addition, *Clymenella* regenerates rather quickly at 15° C., results being obtainable in five to ten days.

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[1] These investigations were made possible partially through a grant from the National Science Foundation, administered by Dr. S. M. Rose, and partially through a National Institutes of Health Predoctoral Fellowship, GM 17,904.

METHODS

General

The worms for these experiments were collected from Barnstable Harbor, Barnstable, Mass., from the entrance to Squeteague Harbor, North Falmouth, Mass., and from the Northwest Gutter entrance to Hadley's Harbor, Naushon Island, Mass., by the M.B.L. Supply Department and by the investigator. Only adult worms, measuring 7–9 cm. in length, were used. For tests of posterior specificity, single segments (17 through 19) were isolated and cultured. For tests of anterior specificity, two-segment pieces (4 and 5, and 6 and 7) were obtained. All test and control pieces were cultured at 15° C. in a solution of 10 mg.% Chloromycetin and 50 units/cc. Mycostatin (E. R. Squibb) in filtered, pasteurized sea water (this solution is hereafter referred to as "treated water"). For the experiments, extracts were prepared by homogenizing the selected tissue in a glass homogenizer, using ice cold treated water as a solvent. These homogenates were then centrifuged at 5000 *g* for ten minutes, to remove large cellular debris, and added immediately to the isolated worm segments in the culture solution. Unless otherwise noted, the varying ratios of tissue to solvent appearing below were devised in an attempt to keep the tissue weight per ml. of solvent as nearly constant as possible for all tissues tested.

Series A. Test for posterior specificity

In this series of experiments, 161 posterior segments were isolated and divided into three groups. Forty-four segments were placed in individual containers (the depressions of an Hutzler ice ball tray) containing 4 ml. of treated water and 1 ml. of an extract prepared in the ratio of 1 prostomial piece (the prostomium was cut off at the level of the mouth, or just anterior to it) to 3 ml. of treated water. Sixty-four isolates were cultured in the same manner in depressions containing 4 ml. of treated water and 1 ml. of an extract of anal collars prepared in the ratio of one collar per ml. treated water (the anal collar of *Clymenella* contains almost exactly one-third as much tissue as the prostomium). The remaining 53 isolates were cultured as controls in 5 ml. of treated water alone. The segments were examined after 10 days.

Series B. Test for dorsal-ventral specificity within the anal collar

Here, 244 posterior segments were isolated. Ninety-eight were cultured in 4 ml. of treated water plus 1 ml. of an extract made from the dorsal halves of anal collars in a concentration of one dorsal half per ml. Ninety-eight others were treated in exactly the same manner, except that the test extract was made from the ventral halves of anal collars in a ratio of one ventral half per ml. treated water. The remaining 48 isolates were placed in 4.5 ml. of treated water plus 0.5 ml. of an extract of 2.4 ventral halves/ml. treated sea water. This slight increase in final extract concentration was obtained to check the possibility that the results of the immediately preceding series of tests with ventral half extracts represented a borderline concentration phenomenon. Examination of the test segments was made after 10 days.

Series C. Test for anterior-posterior gradient of tail-inhibiting substance

(1) In the first group of tests, 28 fifteenth and 28 seventeenth segments, placed in individual trays containing 4 ml. of treated water, received 1-ml. aliquots of an extract of 12 sixteenth segments per 32 ml. of treated water. In this way, the fifteenth segments were treated with material normally posterior to them, and the seventeenth, with material normally anterior to them. (2) In the second group, in order to provide clearer results, material from more distant regions was used, and in higher concentration. Fourteen sixteenth segments, cultured in 4 ml. of treated water, received 1-ml. aliquots of an extract of 14 fourteenth segments in 15 ml. of treated water. Fourteen fifteenth segments, cultured in 4 ml. of treated water, received 1-ml. aliquots of an extract of 14 seventeenth segments in 15 ml. of treated water. The test segments in groups 1 and 2 were cultured at a slightly elevated temperature (19° C.), and the results were therefore tabulated at the end of one week, rather than at the end of ten days.

Series D. Test for anterior specificity

Two hundred seventy two-segment anterior pieces were isolated for these experiments and divided into three groups. Ninety, placed in 4 ml. of treated water, received 1-ml. aliquots of an extract of prostomial pieces (obtained as in Series A) in a concentration of one piece per 3 ml. of treated water. Ninety more were cultured in 4 ml. of treated water plus 1 ml. of an extract prepared by grinding one anal collar per ml. of treated water. The remaining 90 isolates, serving as controls, were allowed to regenerate in 5 ml. of treated water alone. All segments were examined after ten days (cultured at 15° C.).

Series E. Tests for the electrophoretic mobility of the inhibitors

A lucite tray, with inside dimensions of 17.5 cm. × 8.5 cm. × 5 mm., was filled with 2% agar in sea water and prepared as in Figure 1. Three 7.5 cm × 5 mm. × 5 mm. transverse wells (H.W. in Figure 1) were cut into the agar to hold the homogenates to be tested. Four rows of 14 longitudinal wells 9 mm. × 2 mm. × 2 mm. (S.W. in Figure 1) were then cut into the agar 2 mm. away from the transverse wells. Into these wells were placed the test segments. Isolated posterior segments were placed so that their posterior cut surfaces always faced the nearest transverse well. Anterior isolates were arranged with their anterior cut surfaces facing the transverse wells. (This arrangement allowed the regenerating surface to be in the closest possible proximity to the homogenate.) With the segments in place, the longitudinal wells were filled with treated water and then covered with strips of wet knit cotton cloth to retard evaporation and drying of the test segments. The transverse wells were then filled with the appropriate homogenate or solution and covered with strips of polyethylene film. Subsequently, strips of Whatman no. 1 filter paper were moistened with sea water and placed at the ends of the agar tray, with their free ends hanging down. This whole apparatus was then lowered into place in an electrophoresis chamber surrounded with ice (to maintain a temperature of 15°–17° C. for the segments under treatment). The buffer wells were filled with cold sea water. A current

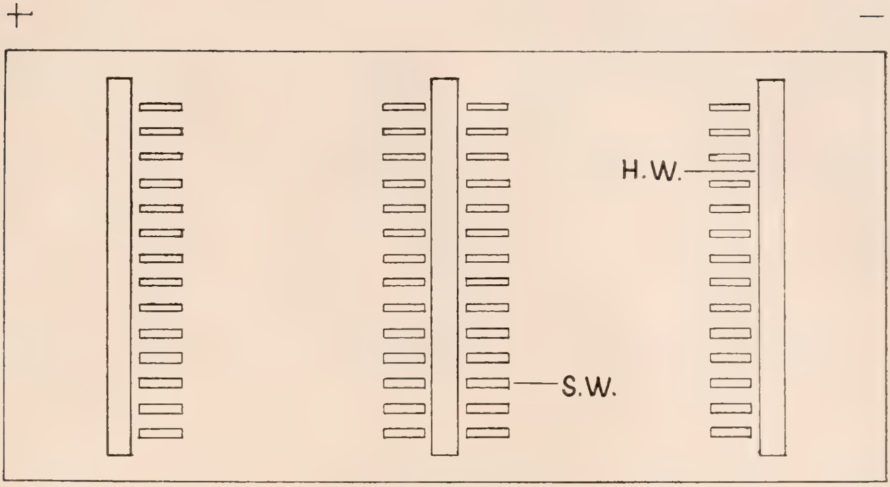

FIGURE 1. Electrophoresis tray; H.W., homogenate well; S.W., test segment well.

of 20 milliamperes at 15 volts was then applied with a VoKam constant current/constant voltage D. C. power supply (COLAB Instrument Co.) for four hours. After the four-hour treatment, the isolated segments were removed to individual trays containing 5 ml. of treated water. The segments were inspected after ten days, and the results were recorded. The experimental arrangements were as follows: (1) One hundred seventy-two posterior segments were divided into three groups. Fifty-six were placed in the apparatus as described above, and the homogenate wells were filled with an extract of 40 anal collars in 4 ml. of treated water (the higher concentrations used in this and the following experiments were selected in order to compensate for any loss of inhibitor during electrophoretic migration, and to ensure a steady supply of it to the test segment for the whole four hours of electrophoresis). Thus, 28 of these 56 segments were left with their posterior surfaces facing the positive pole, and 28 were left with posterior surfaces facing the negative pole. Fifty-six more segments acted as one control, being treated exactly as above, except that the homogenate wells were filled only with the treated water. The final 60 isolates were also treated exactly as above, except that the homogenate consisted of 20 prostomial pieces in 4 ml. of treated water. This last group served as a control against any possible cross-reactivity of the inhibitors. (2) One hundred seventy-two anterior two-segment isolates were also divided into three groups as above and treated with the same series of extracts and solutions in exactly the same concentrations and in exactly the same fashion as the posterior segments except that, as previously noted, the anterior cut surfaces faced the transverse homogenate wells.

RESULTS

Series A

The results of the tests for posterior specificity may be seen in Table I and Figure 2. Of the 53 control segments cultured in plain treated water, 8 had to be discarded due to improper wound closure. (These discards are listed in Table I and in subsequent tables as "out.") In general, approximately 15% of the isolated posterior segments showed this defect. It was a result of the closing over of the posterior end of the gut tube. There followed the protrusion of a large, fluid-filled sac which pressed upon the surrounding tissue and effectively prevented regeneration. Of the remaining 45 controls, 44 produced perfect anal collars in ten days (Fig. 2A). Illustration (B) of Figure 2 shows a typical segment treated with prostomial extract. Of the 40 posterior segments so treated which remained healthy, 36 produced perfect tails, fully as large as those of the controls. The segments treated with anal collar extract, however, presented an entirely different picture, typified by drawing (C) of Figure 2. Not a single one of the segments which closed its wound properly regenerated a tail. The wound merely closed over, and the process stopped at that point. A few segments were allowed to progress for a month, and at the end of that time, the majority showed signs of recovery and regeneration, but within the limits of the experiment, no recovery was evident.

TABLE I

Posterior specificity

Treatment	No. cases	No. out	No. regenerates	% Regenerates
Control	53	8	44/45	97.7
Prostomial extract	44	4	36/40	90.0
Anal collar extract	64	10	0/54	0.0

Series B

The results of this series of experiments are presented in Table II and Figure 3. The results, in this case, were somewhat less striking than in the previous series. Of the 98 posterior segments treated with dorsal collar extracts, 9 closed wounds improperly and were discarded. Twenty-nine of the remaining 89 pieces showed either no regeneration at all, or only slight accumulations of new tissue within the limits of the experiment. The remaining 60 pieces, however, when compared with a control segment ('A' of Figure 3) from the previous series, presented interesting results. As may be seen from part (B) of Figure 3, the dorsal segment of the regenerating anal collar was much reduced and lacked cirri. The situation which prevailed with the posterior segments receiving ventral collar extracts was much the same in nature as that above, except that the incidence of homologous inhibition was much lower, and the incidence of total inhibition much higher. Of the 98 segments treated with the 1:1 ventral collar extract, 13 were discarded. Forty showed homologous inhibition of the ventral half of the anal collar ('C' of Figure 3). Forty-five, however, were completely inhibited. These data suggested that perhaps the difference between the specific and non-specific total inhibitions might be a matter of concentration. Therefore, the second group of ventral collar extract tests was run with slightly higher final concentration (1.2:1). Of the 48 segments so treated, 7 were discarded, only

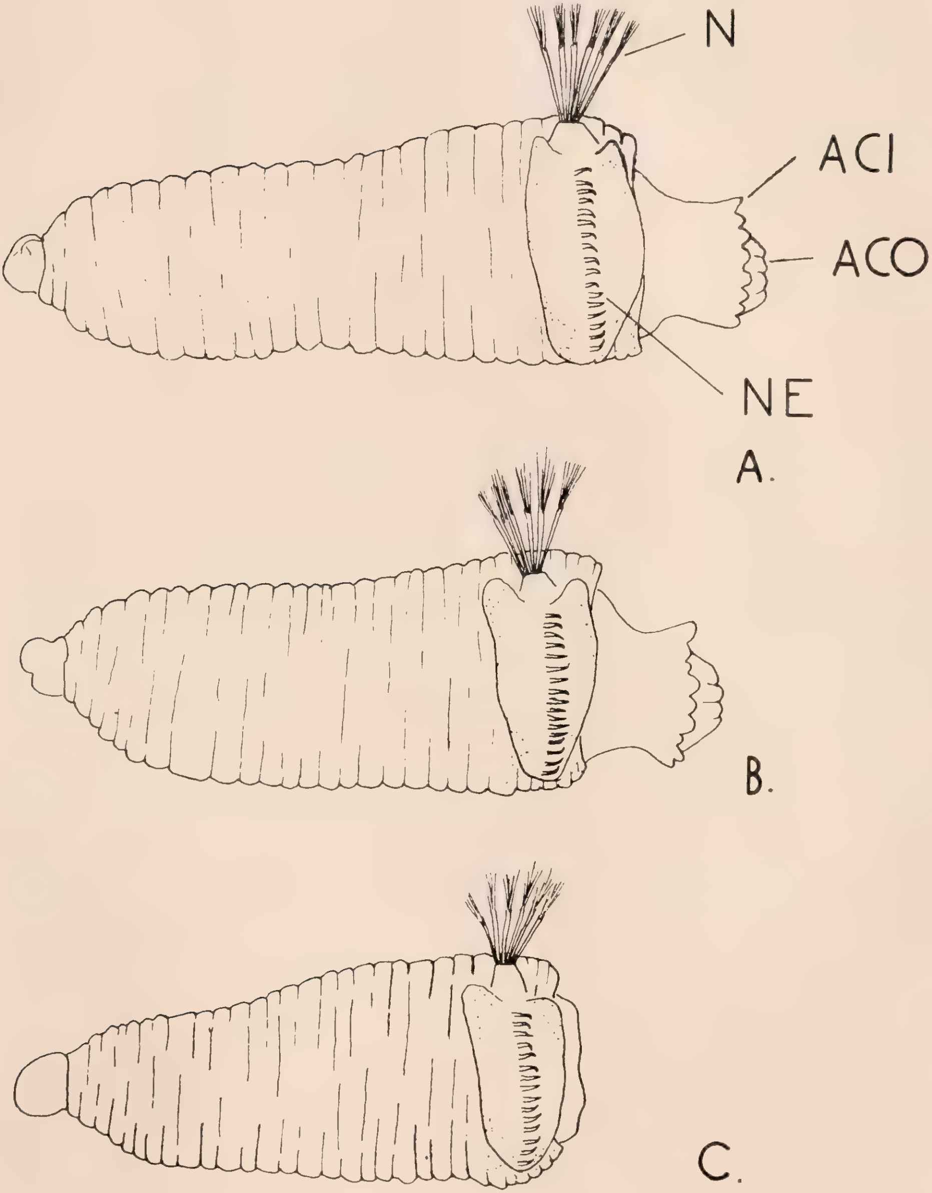

FIGURE 2. A. Posterior segment treated with culture water only. N, notosetae; NE, neurosetae; ACI, anal collar with cirri; ACO, anal cone (pygidium). B. Posterior segment treated with prostomial extract. C. Posterior segment treated with anal collar extract.

TABLE II

Dorsal-ventral specificity

Treatment	No. cases	No. out	No. totally inhibited	No. dorsally inhibited	No. ventrally inhibited
Dorsal collar extract	98	9	29	60	0
Ventral collar extract 1:1	98	13	45	0	40
Ventral collar extract 1.2:1	48	7	28	0	13

13 were homologously inhibited with reduced ventral segment, and the rest were totally inhibited.

Series C

Of the original 28 posterior segments treated with materials from the segment immediately posterior to them, 22 closed their wounds properly. Of these, only four proceeded to initiate regeneration, and this was so slow and late that within the seven-day limit of the experiment, no indications of normal anal collars and cirri appeared. The 28 pieces treated with extract of the immediately preceding segment presented a curious phenomenon. Fifteen of these pieces began to develop a peculiar blistering of the epidermis at the anterior end after two days. This condition progressed posteriorly and inwardly during the following five days, arriving finally at a point where the entire test segment appeared to be a mere sac of dissociated cells. These pieces did not regenerate. However, the remaining 13 test segments, though they began to show signs of the same affliction, proceeded to regenerate anal collars normally.

The 14 test pieces (segment 15) treated with extracts of segment 17 were inhibited without exception. One piece was discarded, and of the remaining 13, none developed new tissue within the seven-day period. One of the 14 sixteenth segments treated with segment 14 extracts was discarded. Five developed the blistering reaction noted above, but the eight which were left regenerated normally.

Series D

The 90 control segments of this series regenerated uniformly, 80 outgrowths, averaging 0.8 mm. in length, appearing, with only 10 failures. In this case, the failures were due to the fact that the isolating cuts were not precisely in the intersegmental plane. Under such conditions, regeneration failed. The appearance of a control segment may be seen in Figure 4A. Figure 4B and Table III show that

TABLE III

Anterior specificity

Treatment	No. cases	No. regenerates	No. failures
Control	90	80	10
Prostomial extract	90	15 (delayed)	75
Anal collar extract	90	76	14

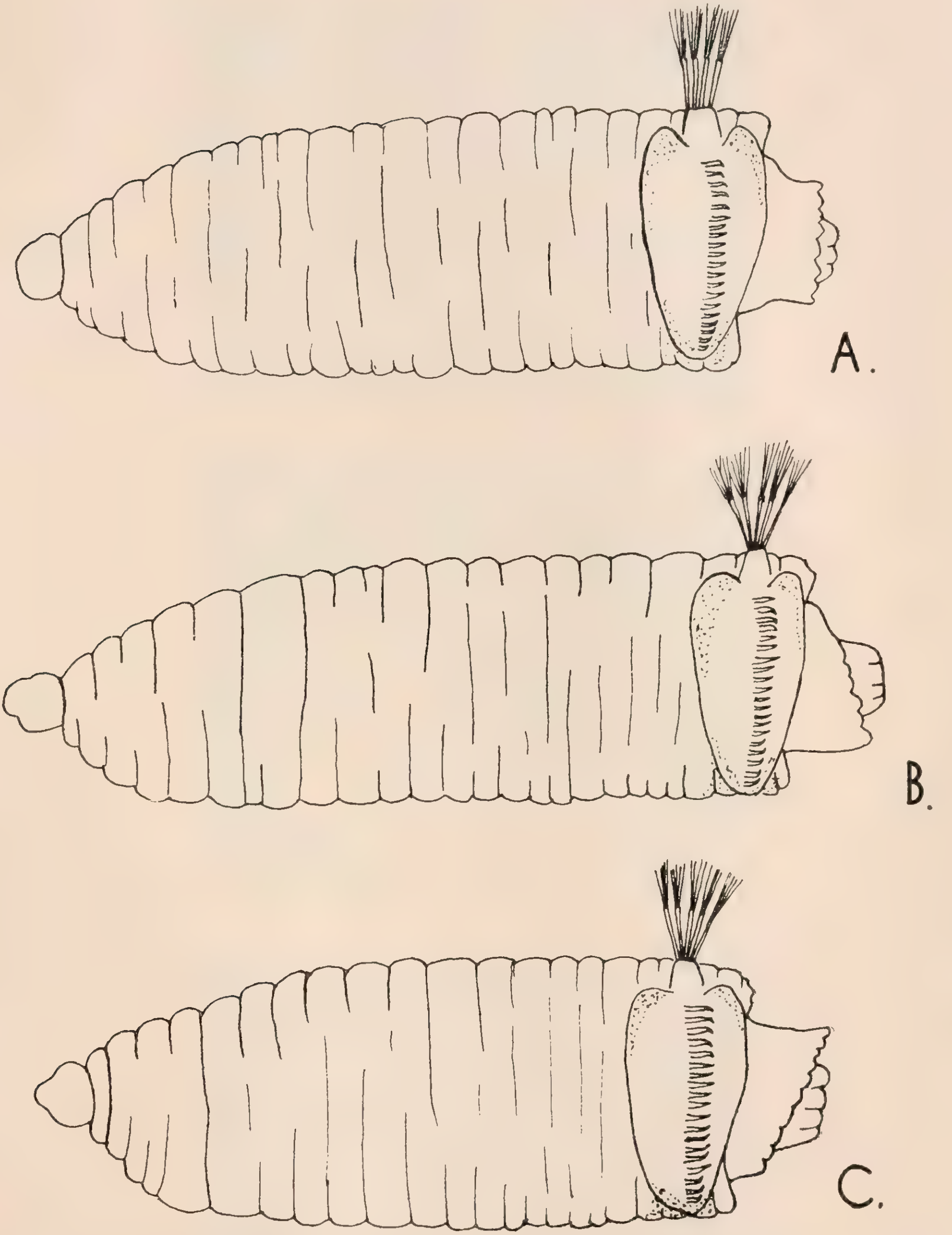

FIGURE 3. A. Posterior segment treated with culture water only. B. Posterior segment treated with dorsal collar extract. C. Posterior segment treated with ventral collar extract.

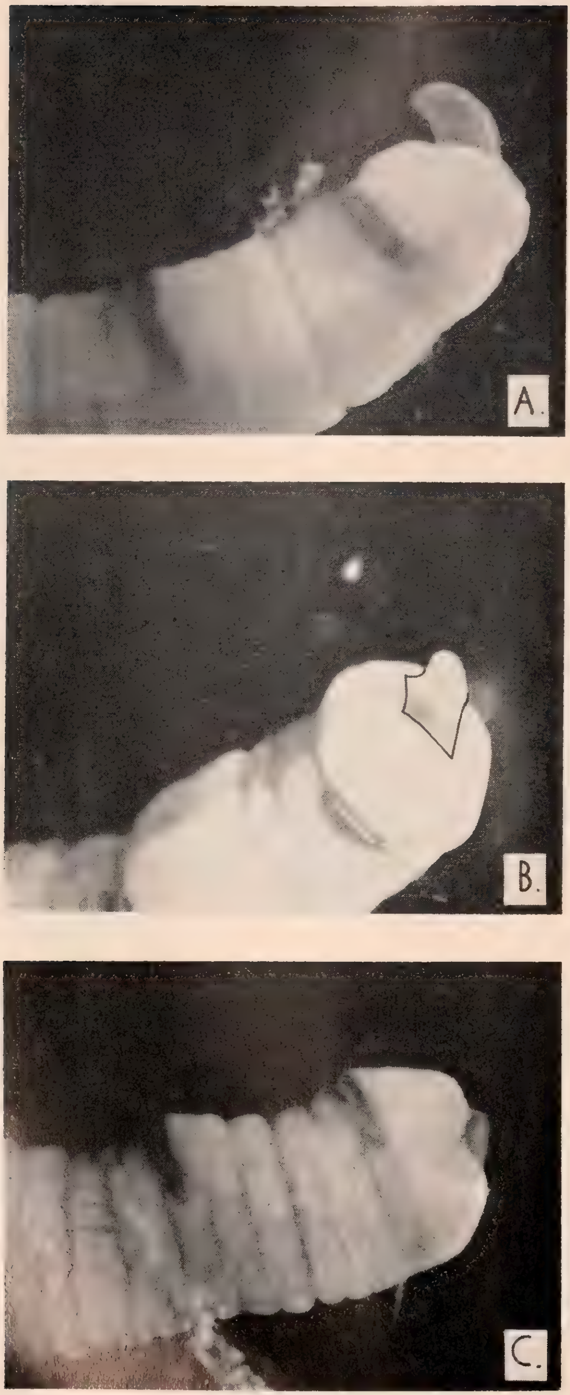

FIGURE 4. A. Anterior segments treated with culture water only. B. Anterior segments treated with anal collar extract. C. Anterior segments treated with prostomial extract.

the 90 pieces treated with tail extract regenerated almost as well as the controls. Seventy-six regenerated and 14 failed, presumably because of improper cutting. The 90 test pieces treated with prostomial extract fared very poorly. Only 15 of the 90 produced regenerates, and these were very small, none of them exceeding 0.2 mm. in length. Seventy-five failed to regenerate at all, though 10 to 15 of the failures were no doubt the result of improper cuts. Figure 4C shows one of these failures.

Series E

The results of the tests for electrophoretic mobility of the inhibitors may be seen from Table IV and Figures 5 and 6. Of the 172 posterior segments placed in the agar trays, only the 28 with the posterior cut surface facing the positive pole and a well filled with anal collar extract showed any evidence of specific inhibition. The other 144 were essentially unimpaired by the procedures and regenerated normally. Of course there were some failures due to improper wound

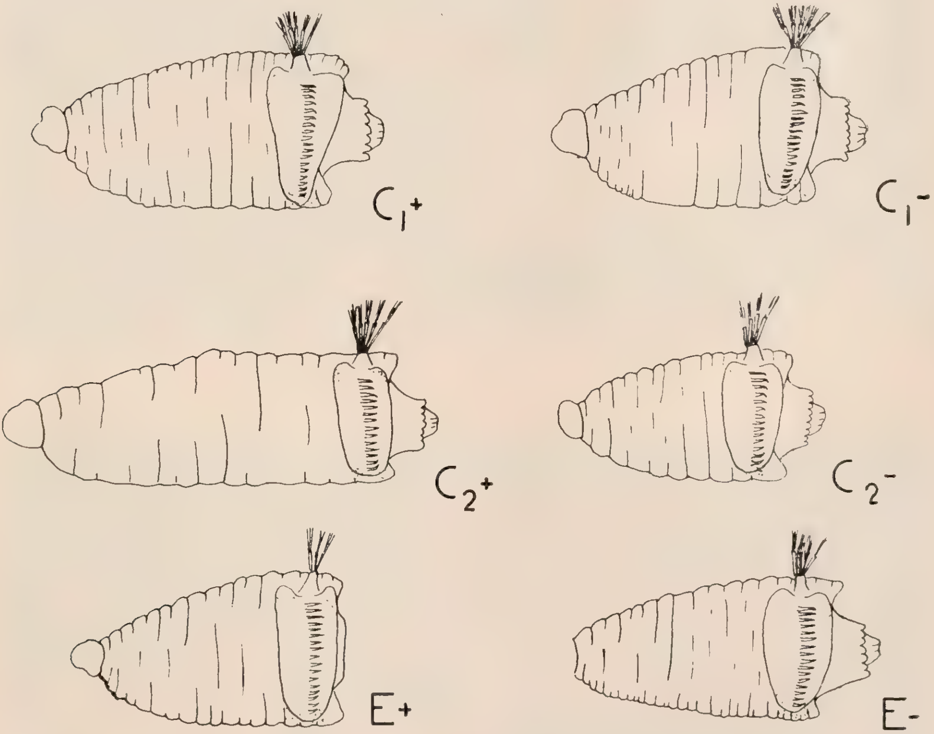

FIGURE 5. C_1+, posterior segment, posterior cut surface facing positive pole, treated water in homogenate well. C_1-, posterior segment, posterior cut surface facing negative pole, treated water in homogenate well. C_2+, posterior segment, posterior cut surface facing positive pole, prostomial extract in homogenate well. C_2-, posterior segment, posterior cut surface facing negative pole, prostomial extract in homogenate well. E+, posterior segment, posterior cut surface facing positive pole, anal collar extract in homogenate well. E−, posterior segment, posterior cut surface facing negative pole, anal collar extract in homogenate well.

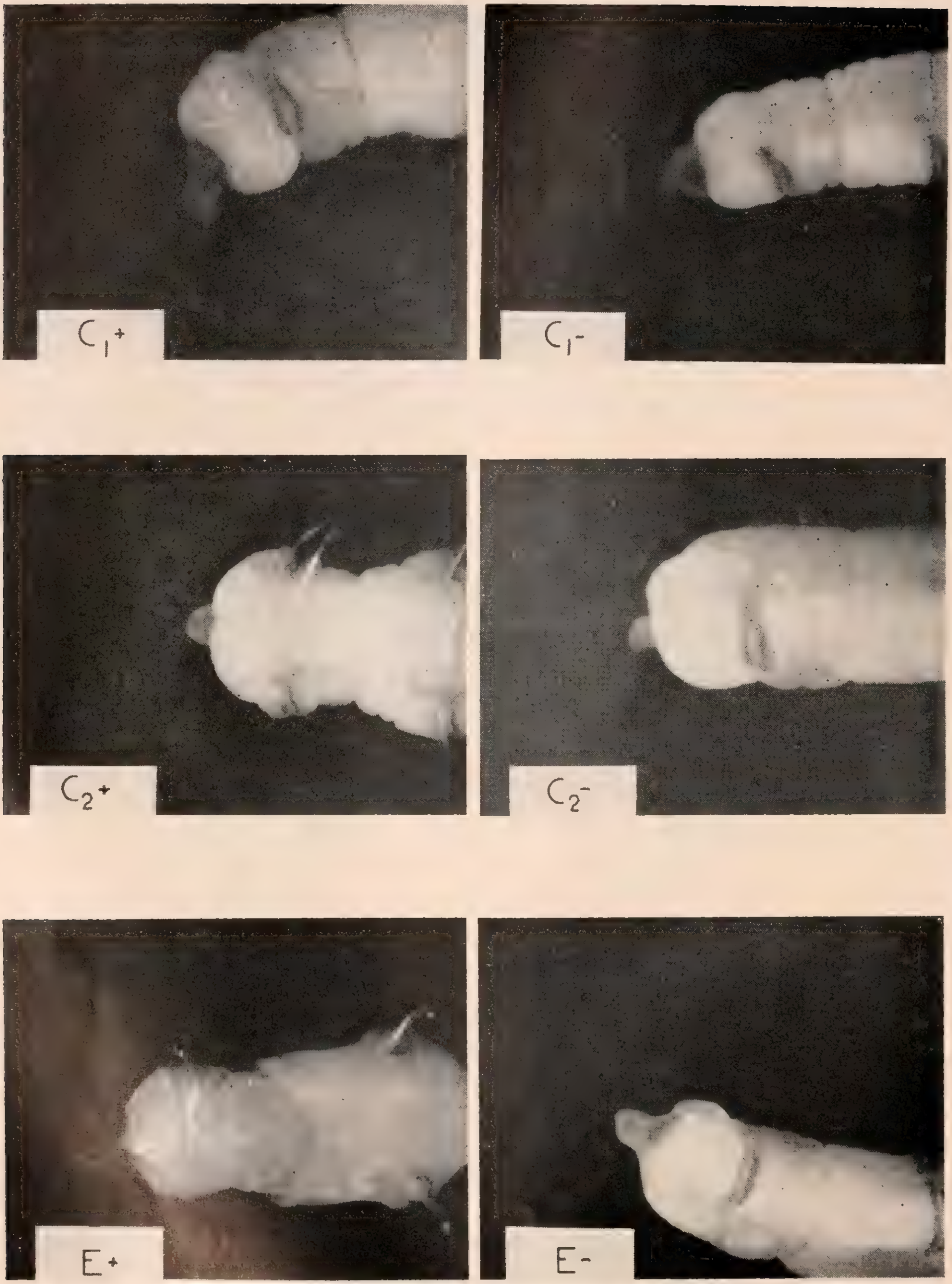

FIGURE 6. C_1+, anterior segments, anterior cut surface facing positive pole, treated water in homogenate well. C_1-, anterior segments, anterior cut surface facing negative pole, treated water in homogenate well. C_2+, anterior segments, anterior cut surface facing positive pole, anal collar extract in homogenate well. C_2-, anterior segments, anterior cut surface facing

TABLE IV

Electrophoretic mobilities

Segment	No. cases	Cut surface facing +	Cut surface facing −	Extract	No. out	No. regenerates	No. failures
Posterior	28	posterior	anterior	water	1	26	1
Posterior	28	anterior	posterior	water	1	27	0
Posterior	30	posterior	anterior	prostomial	3	24	3
Posterior	30	anterior	posterior	prostomial	2	26	2
Posterior	28	posterior	anterior	an. collar	0	2	26
Posterior	28	anterior	posterior	an. collar	1	25	2
Anterior	28	anterior	posterior	water	1	24	3
Anterior	28	posterior	anterior	water	3	22	3
Anterior	30	anterior	posterior	an. collar	0	27	3
Anterior	30	posterior	anterior	an. collar	2	27	1
Anterior	28	anterior	posterior	prostomial	0	0	28
Anterior	28	posterior	anterior	prostomial	1	26	1

closure. Similar results were obtained with the anterior test pieces. The 28 with anterior cut surfaces facing the positive pole and a well filled with prostomial extract showed total inhibition. The other 144 were unaffected (Fig. 6E).

DISCUSSION

The results of these investigations are relatively clear, and may be readily interpreted, except for one or two points. The results of the tests for anterior and posterior specificity may be interpreted as demonstrating the presence of two distinct moieties. One, produced in the anal collar, can specifically inhibit its regeneration, and presumably acts in a similar capacity to limit its size in normal development. A similar specific moiety may be postulated as arising in the prostomium during development, to check its growth. When extracted, this substance is also capable of checking head regeneration. Marie Tucker (1959) has demonstrated the presence of a similar pair of extractable head- and tail-inhibitors which are specific for their homologous structures in the nemertean, *Lineus vegetus*. Lender (1956) describes an inhibitor which is specific for the brain of *Dugesia lugubris*. He points out that tail extracts have no effect on the regeneration of this structure. The data gathered in these investigations almost certainly represent evidence of the same sort of phenomenon.

The results of the tests for dorsal-ventral specificity within the anal collar strongly suggest that the tail-inhibitor may be divided into at least two distinct inhibitors, one of which limits dorsal development, and one of which affects the ventral half of the collar. However, the high incidence of total inhibition with dorsal, and especially with ventral, extracts casts some doubts. It may perhaps be true that the dorsally and ventrally evolved inhibitors are quite specific in their actions. If this be the case, then one must postulate the presence of a third and

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negative pole, anal collar extract in homogenate well. E+, anterior segments, anterior cut surface facing positive pole, prostomial extract in homogenate well. E−, anterior segments, anterior cut surface facing negative pole, prostomial extract in homogenate well.

more general inhibitor present in slightly lower concentration, to explain the increase in total inhibition with a slight increase in extract concentration. Perhaps a more likely explanation of the observed facts would assume that the dorsal and ventral inhibitors possess, in addition to their specific action, the ability to cross-react with their opposite halves if present in sufficiently high concentration. In any event, this dichotomy of possibilities exists and awaits further elucidation.

The results of the tests for an anterior-posterior gradient of tail-inhibiting substance provide evidence of two phenomena. Material from posterior segments, when placed in contact with more anterior segments, prevents them from regenerating a tail. Material from a level normally more anterior than the test segment does not inhibit tail regeneration. These facts demonstrate nearly conclusively the presence of a gradient of tail-inhibitory activity with its zenith at the posterior end of the worm. There is apparently also a gradient in the opposite direction. The large number of segments showing the blistering-disaggregation reaction when treated with extracts of a more anterior region, but not when treated with more posterior material, is a very interesting observation. One is tempted to postulate an anterior-to-posterior gradient of some kind of somatic tissue factor—perhaps the sort of factor which may determine "headness" and "tailness" in the worm, and or which, by virtue of some sense of quantitative distribution, allows the worm to maintain its almost universally constant number of 22 segments. However, this obviously remains to be proved.

The results of the electrophoretic mobility tests clearly indicate that at sea water pH (*ca.* 8.1) both the head- and tail-inhibitors are positively charged. They move through the agar toward the negative pole and apparently enter the exposed surfaces of the test pieces. The electrical current itself obviously has no effect, nor is there any cross-reaction of the inhibitors. There is, of course, no valid reason for concluding that this positive charge is present within the worm's tissues, the pH there being unknown. Nor is it known that the two inhibitors possess the same degree of positivity. The possibility is apparent, however, that the inhibitors may be electrophoretically mobile within the worm. It would be very interesting to know what, if any, natural bioelectric polarities exist within this species, since electrical fields have been shown to be capable of controlling the polarity of growth in *Fucus* (Lund, 1923), *Obelia* (Lund, 1925), *Dugesia* (Marsh and Beams, 1952), and *Perophora* (Smith, unpublished data). Bioelectric fields have also been implicated in the control of amphibian limb regeneration (Becker, 1961). If one could correlate the posterior-anterior gradients of inhibitory activity with naturally occurring bioelectric fields, it would suggest that pattern and growth specificities might be due to the specific mobilization and concentration of substances within such fields.

SUMMARY

1. Evidence is presented suggesting the presence of specific head- and tail-inhibitors, and of even more specific dorsal-ventral intra-organ tail-inhibitors.

2. The tail-inhibitor is shown to be distributed along a posterior-to-anterior gradient.

3. Another substance, a "somatic factor," is postulated as existing along an anterior-to-posterior gradient.

4. The electrophoretic mobilities of the head- and tail-inhibitors at sea water pH are demonstrated.

5. The possibility of bioelectric control of the movements and concentrations of growth- and pattern-specific substances is briefly considered.

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SYNTHESIS AND TRANSFER OF DNA, RNA, AND PROTEIN DURING VITELLOGENESIS IN RHODNIUS PROLIXUS (HEMIPTERA)[1]

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Two tissues, the apical trophocytes and the follicular epithelium, are active in transferring yolk to the growing oocytes in the ovarioles of most insects. The telotrophic ovary of Hemiptera is particularly useful in the study of vitellogenesis because the two nutritive tissues are sufficiently set apart so that the special activity of each can be separately investigated. Of especial interest is the study of the nucleic acid and protein components of the oocyte yolk. Investigations on the origin, distribution and nature of these materials are of fundamental importance in the search toward an understanding of the biochemistry of insect embryology.

Important cytochemical studies on vitellogenesis in Hemiptera have been performed by Schrader and Leuchtenberger (1952), who showed the apparent transfer of DNA in a depolymerized form from the apical trophocytes to the oocytes in the coreid bug, *Acanthocephala;* and by Bonhag (1955), who showed the passage of nucleic acids and protein from the trophocytes to the oocytes in the ovary of the milkweed bug, *Oncopeltus.*

The autoradiographic technique has distinct advantages over the cytochemical approach in the study of a dynamic process such as the transfer of materials between tissues. Instead of being restricted to merely localizing a chemical substance in a tissue section, it becomes possible to determine its site of synthesis, and to trace its movement from one tissue to another.

The present paper attempts to define more precisely the role of the trophic tissues in the genesis of the nucleic acid and protein components of the yolk in the growing insect oocyte, by utilizing the autoradiographic technique in conjunction with cytochemistry.

MATERIALS AND METHODS

Mature *Rhodnius prolixus* adult females were used exclusively in the experiments. The bugs were maintained at 28° C. in a high humidity cabinet. They were fed weekly on the shaven belly of an immobilized rabbit.

A. Histological and cytological methods

The bugs being studied were vivisected under physiological saline solution. The ovaries and portions of the midgut and ventral fat body were dissected out and

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fixed in Carnoy's (6:3:1) fixative for 45 minutes, or in alcoholic Bouin's solution for 24 hours. Mature oocytes with a well developed chorion were punctured with a fine needle in order to facilitate penetration of histological reagents.

After fixation, Carnoy-fixed tissues were washed for one hour in absolute ethanol, while Bouin-fixed tissues were washed in several changes of 80% ethanol over a three-day period. The tissues were then hydrated to distilled water via a graded series of alcohol concentrations. Wigglesworth's (1959) agar-tested

Plate I

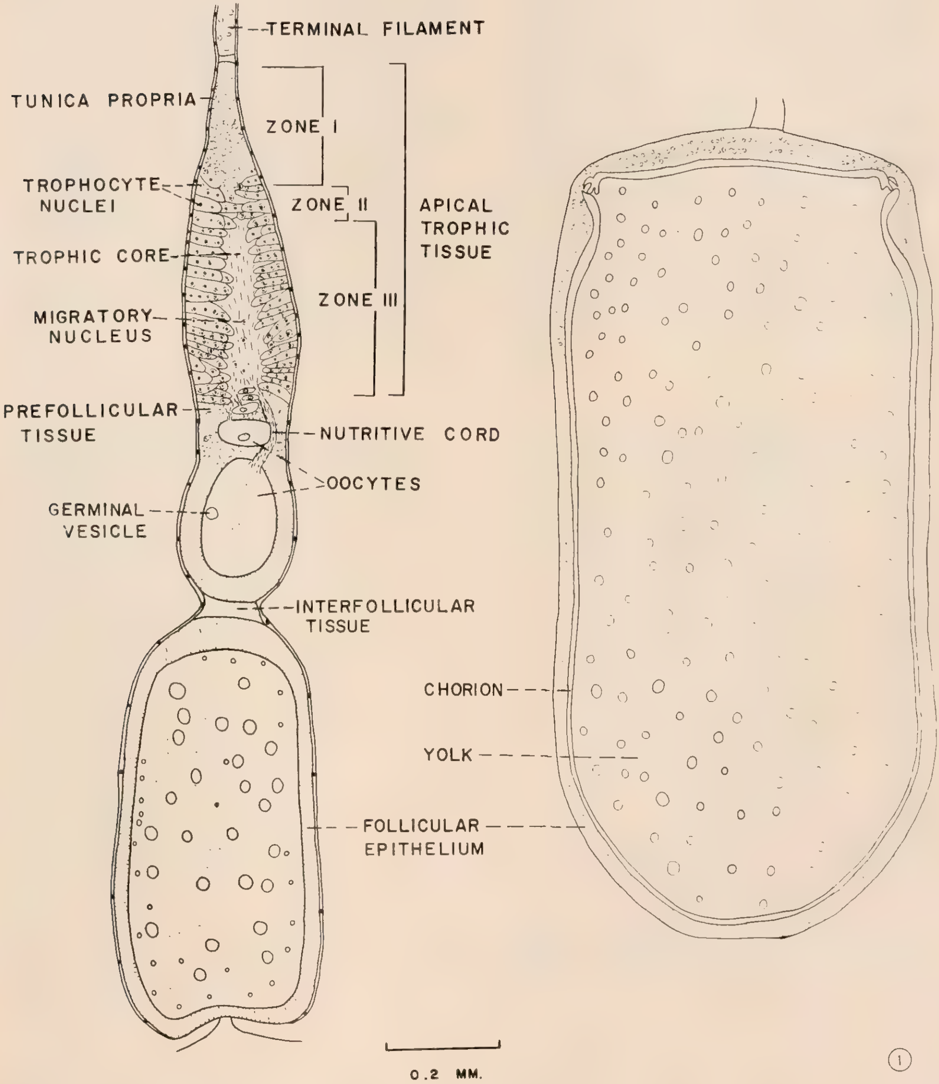
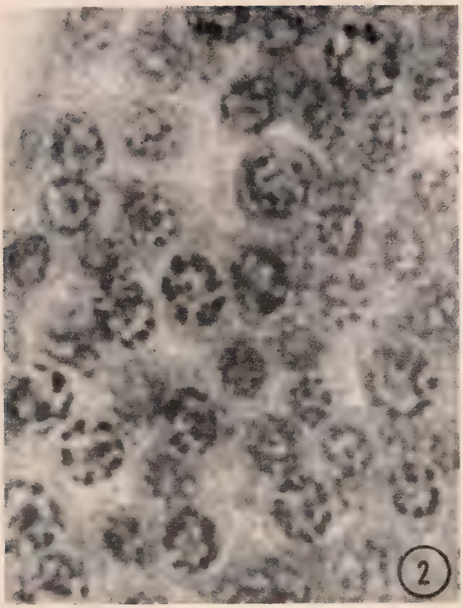
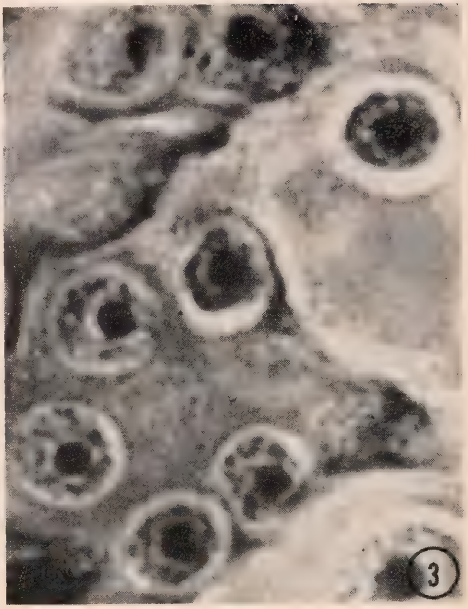
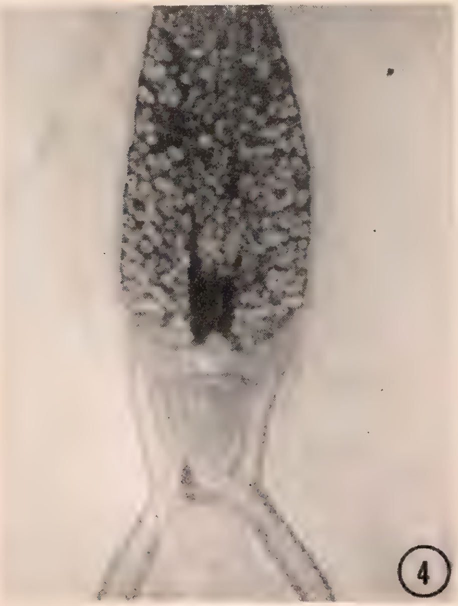

TERMINAL FILAMENT
TUNICA PROPRIA
ZONE I
TROPHOCYTE NUCLEI
ZONE II
APICAL TROPHIC TISSUE
TROPHIC CORE
ZONE III
MIGRATORY NUCLEUS
PREFOLLICULAR TISSUE
NUTRITIVE CORD
OOCYTES
GERMINAL VESICLE
INTERFOLLICULAR TISSUE
CHORION
YOLK
FOLLICULAR EPITHELIUM
0.2 MM.
①

Figure 1. Sagittal view of *Rhodnius* ovariole.

PLATE II







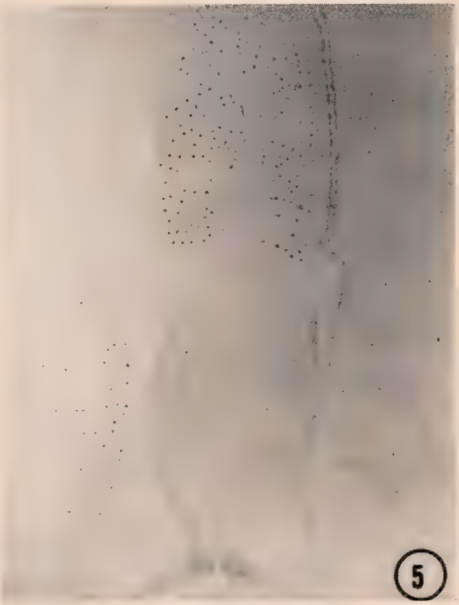


FIGURE 2. Zone I cells of apical trophic tissue. The large nuclei contain peripheral chromatin granules. Heidenhain's hematoxylin. Approximately 1250 ×.

FIGURE 3. Zone II of apical trophic tissue. The enlarged nuclei now have centrally located nucleoli, and are located in clumps of cytoplasm. Heidenhain's hematoxylin. Approximately 1325 ×.

wax double embedding technique was utilized to prepare serial sections of 5- to 7-micron thickness. These sections were stained with Heidenhain's iron hematoxylin. They were differentiated in a saturated solution of picric acid in 70% alcohol, and no counterstain was employed.

B. Histochemical methods

The localization of DNA was accomplished by the Feulgen method used as follows. Carnoy-fixed sections were hydrolyzed in 1.0 N HCl at 60° C. for 8 minutes, while adjacent sections were placed in distilled water as controls. Sections were then rinsed in distilled water and transferred to the Schiff reagent at room temperature for one hour. They were then rinsed in three changes (10 minutes each) of freshly prepared bleaching solution consisting of 5 ml. of 10% aqueous $K_2S_2O_5$, 5 ml. of 1.0 N HCl, and 90 ml. of distilled water. This was followed by washing in tap water for 5 minutes, dehydration, clearing, and mounting in balsam.

RNA was detected by staining Carnoy-fixed sections with a potassium acid phthalate-buffered solution of Azure B bromide following exactly the procedure developed by Flax and Himes (1952). Adjacent control sections were pretreated with ribonuclease[3] (0.5 mg./ml. glass-distilled water) at 37° C. for two to four hours, while the test sections were incubated in distilled water at the same temperature.

C. Autoradiographic methods

The autoradiographic localization of sites of incorporation of tritium-labeled precursors into DNA, RNA, and protein was determined as follows. The bugs being studied were each injected with 5 microcuries of thymidine-H^3 (1900 mc./m*M*) for determination of DNA synthesis, 5 microcuries of uridine-H^3 (3280 mc./m*M*) for determination of RNA synthesis, or 10 microcuries of DL-leucine-4,5-H^3 (3570 mc/m*M*) for determination of protein synthesis. The radioactive solutions were so diluted that each bug received 0.01 ml. per injection.

An uptake time of 15 minutes to two hours was allowed, after which the bug was dissected. The tissues were fixed for 45 minutes in Carnoy's solution, hydrated and then thoroughly washed in running water to remove the unincorporated labeled precursors. Using the histological procedures previously described, sections were cut at 7 microns and affixed to glass slides. The slides with hydrated tissues were then washed in water to ensure removal of any remaining labeled precursor. Kodak Nuclear Track Bulk Emulsion (Type NTB-3) was liquefied in a 43° C. water bath, and each slide was dipped in the emulsion and allowed to gel in a vertical postion in a darkroom maintained at 15° C. After

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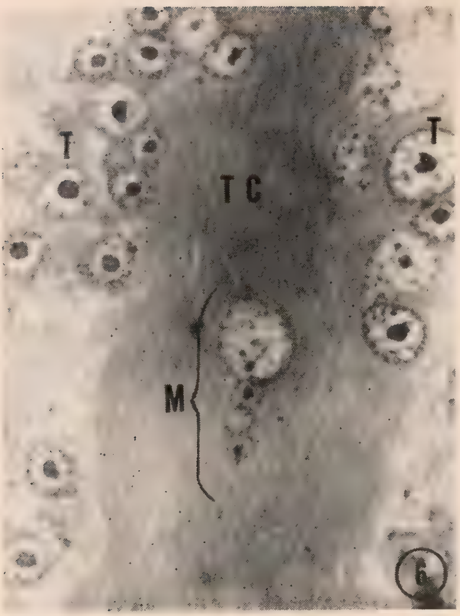
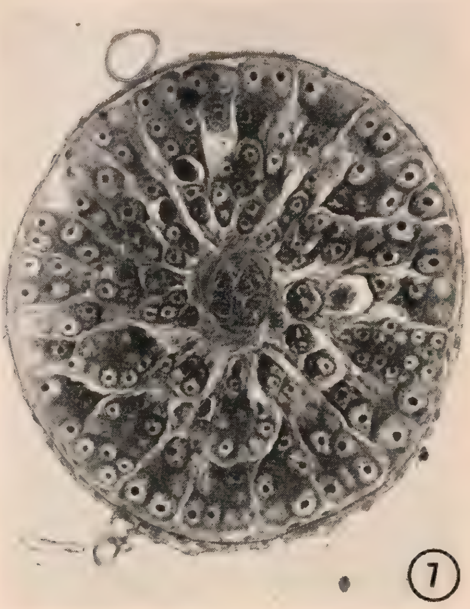
[3] Obtained from Worthington Biochemical Laboratories, Freehold, N. J.

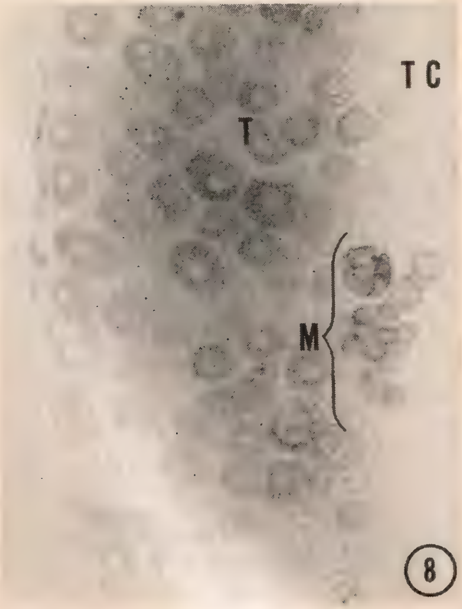
FIGURE 4. Germarium and upper portion of vitellarium showing cytoplasmic basophilia. Azure B bromide. Approximately 100 ×.

FIGURE 5. Section of different ovariole from the same ovary used for Figure 4. Note the loss of cytoplasmic basophilia after two hours of ribonuclease incubation. Nucleolar basophilia still persists in most of the trophocytes. Longer treatment with ribonuclease yields slides that are completely blank and non-photogenic. Azure B bromide. Approximately 100 ×.

PLATE III







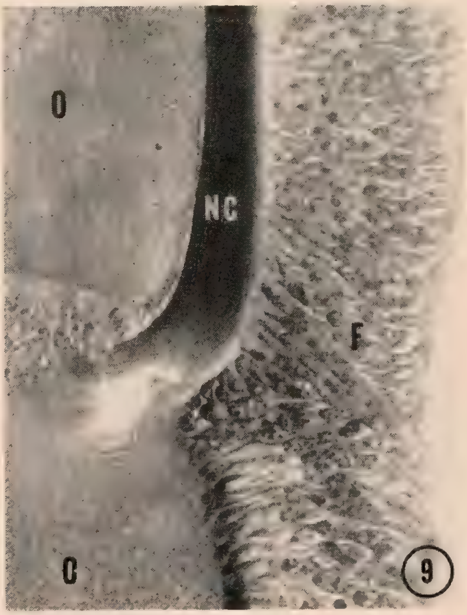


FIGURE 6. Migratory nuclei (M) in trophic core (TC) of Zone III of apical trophic tissue. (T) trophocyte nuclei of Zone III. Heidenhain's hematoxylin. Approximately 800 ×.

FIGURE 7. Zone III of germarium. Cross-section showing masses of cytoplasm projecting radially into trophic core. Heidenhain's hematoxylin. Approximately 250 ×.

drying, the slides were placed in light-tight Bakelite boxes containing Drierite, and allowed to expose for periods of two to four weeks at 3° C. The slides were then developed in Kodak Dektol for 1½ minutes, fixed for 10 minutes in Kodak acid fixer, and washed in several changes of water for 15 minutes. The sections were stained with Azure B bromide as previously described, dehydrated, and mounted in balsam under a cover glass. When viewed under a microscope, those areas of the emulsion with granulation denser than the background presumably lie above areas of the section which were sites of incorporation of the labeled precursor into the macromolecule being studied.

In subsequent experiments, after the initial ½-hour uptake of the labeled precursor, the bug was injected with 100 times the concentration of the same precursor in its unlabeled form. This had the effect of diluting the labeled material to an extent that one could assume only negligible radioactive uptake after this second injection. Bugs so treated were killed and fixed at various time intervals, thus allowing the tracing of the ½-hour "pulse" of radioactivity through the cells.

RESULTS

A. Histology and histochemistry of the ovary

Each ovary of *Rhodnius* consists of seven telotrophic ovarioles. Apically, each ovariole tapers to form a long terminal filament. Immediately posterior to the terminal filament is a lanceolate structure, the germarium, which contains apical trophic tissue, young oocytes, and prefollicular tissue. Posterior to the germarium is the vitellarium, consisting of a string of growing oocytes, each one surrounded by follicular cells. The entire ovariole is enclosed in a membrane, the tunica propria. After the chorion is deposited, the mature oocyte is released from its follicle into the pedicel of the ovariole, from which it passes into the lateral oviduct. Figure 1 is a diagram of a sagittal section of the germarium and vitellarium.

The apical trophic tissue of the germarium may be divided into three zones similar to the zones described by Bonhag (1958) for *Oncopeltus.* In Zone I (Fig. 2), the trophocytes (nurse cells) have distinct boundaries. Each cell contains a single large nucleus with peripheral Feulgen-positive chromatin granules. Occasional mitoses are observed in the region.

Zone II is the transitional zone of trophocyte differentiation. It is marked by the disappearance of individual cell boundaries, the enlarged nuclei coming to lie in clumps of cytoplasm (Fig. 3). Each nucleus possesses a large, centrally located basophilic nucleolus. The Feulgen granules arranged around the periphery of each nucleus are much more intense than those of Zone I, suggesting that these cells are polyploid. The cytoplasm of this region is strongly basophilic. The complete loss of this basophilia after two hours of ribonuclease treatment indicates the presence of RNA (Figs. 4 and 5). The nucleolar basophilia is

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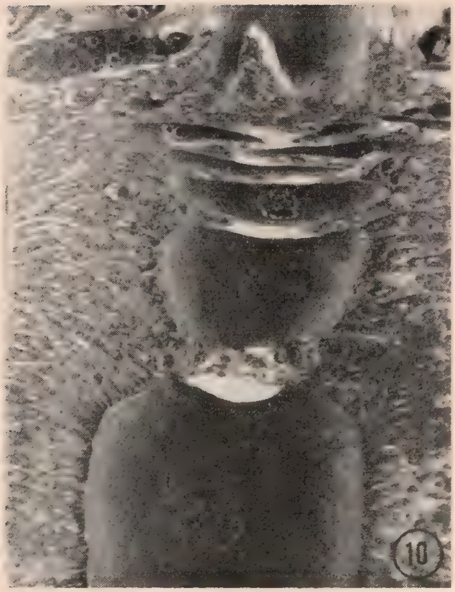
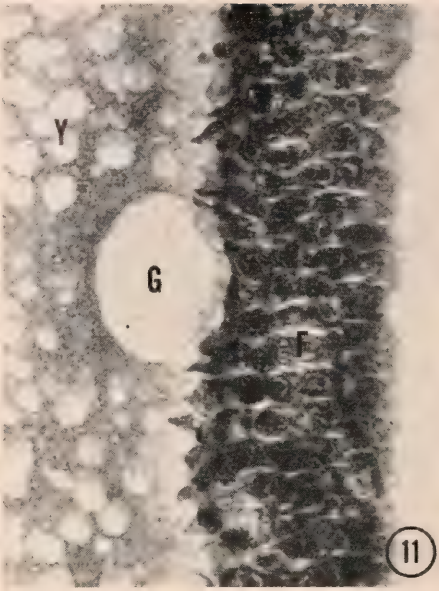
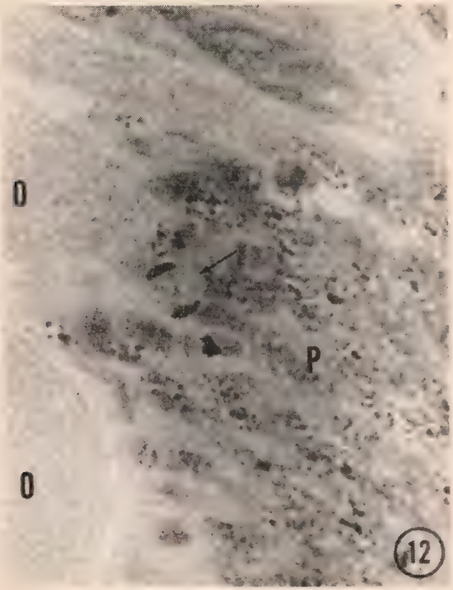
FIGURE 8. Feulgen-stained portion of Zone III of germarium showing (T) trophocytes with Feulgen-positive granules in nuclei, but with Feulgen-negative nucleoli; (M) migratory nuclei in various stages of fusion and breakdown; (TC) trophic core. Approximately 800 ×.

FIGURE 9. Nutritive cord (NC) leading to oocyte (O). (F) follicle cells. Heidenhain's hematoxylin. Approximately 600 ×.

Plate IV







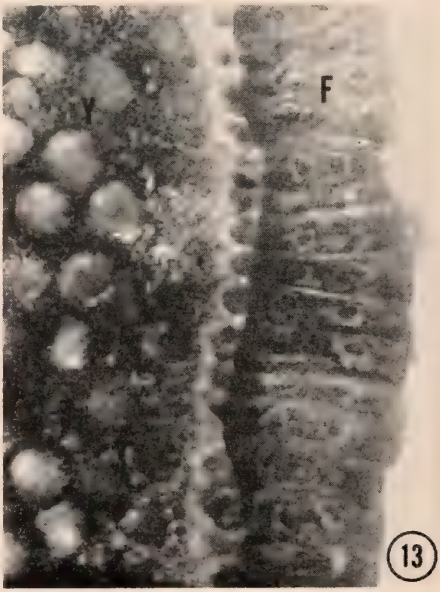


Figure 10. Stages in growth of oocytes. Heidenhain's hematoxylin. Approximately 375 ×.

Figure 11. Germinal vesicle (G) in close proximity to cells of follicular epithelium (F). (Y) yolk. Heidenhain's hematoxylin. Approximately 675 ×.

more persistent, requiring at least four hours of ribonuclease incubation before it is completely lost.

The processes of nuclear aggregation within common cytoplasmic masses continue into Zone III. The nuclei in this region of the germarium are arranged around a central cylinder of cytoplasm, the trophic core. This core appears fibrous in structure, and stains strongly with Azure B bromide (Figs. 4, 5 and 6).

The trophocyte nuclei are imbedded in fingerlike masses of cytoplasm which project radially into the trophic core (Fig. 7). The nuclei appear to be proliferated from these masses, eventually passing into the core. The appearance of migratory nuclei of different sizes within the core (Fig. 6) suggests that some of these nuclei have become fused. The migratory nuclei can be seen in what appear to be stages of degeneration and breakdown, leading to the release of Feulgen-positive material into the core (Fig. 8).

The posterior end of the trophic core connects with the young oocytes by means of cytoplasmic strands, the nutritive cords (Fig. 9). These cords have the same histological and histochemical characteristics as the trophic core. Although present in the posterior portion of the trophic core, neither broken down portions of the migratory nuclei, nor Feulgen-positive material were ever observed in the cords leading to the oocytes.

The oocytes get progressively larger as they pass down from the base of the germarium through the vitellarium (Fig. 10). The germinal vesicle, which also enlarges during oocyte growth, contains Feulgen-positive granules, in addition to basophilic nucleoli-like structures that disappear after ribbonuclease treatment. Vacuoles are generally present. The germinal vesicle is often found in close proximity to the follicular epithelium (Fig. 11). The oocyte yolk, which stains strongly with Azure B bromide during the early stages of development, diminishes in the intensity of its basophilia as the oocyte grows.

Immediately posterior to the apical trophic tissue, and surrounding the young oocytes, is a region of prefollicular tissue (Fig. 12). This is a zone of active mitosis. Cells proliferated from this mass of tissue become oriented around the enlarging oocytes as they pass down the vitellarium. These follicle cells surrounding the young oocytes continue mitotic division during the early stages of oocyte growth.

When the oocyte reaches a size of about 0.4 mm. in diameter, the follicle cells surrounding it undergo amitosis and become binucleate. The follicular epithelium at this stage is made up of tightly packed columnar cells (Fig. 13). As the process of oocyte growth continues, the follicle cells become cuboidal. Each nucleus gives an intense Feulgen reaction, suggesting that it is polyploid (Fig. 14). The strongly basophilic cytoplasm, with no staining in the ribonuclease controls, indicates the presence of RNA (Figs. 15 and 16).

After the completion of yolk deposition, the follicle cells lay down the various layers of the chorion and then by means of prominent villi imprint upon it the characteristic surface sculpturing (Fig. 17). The cytology of the follicular

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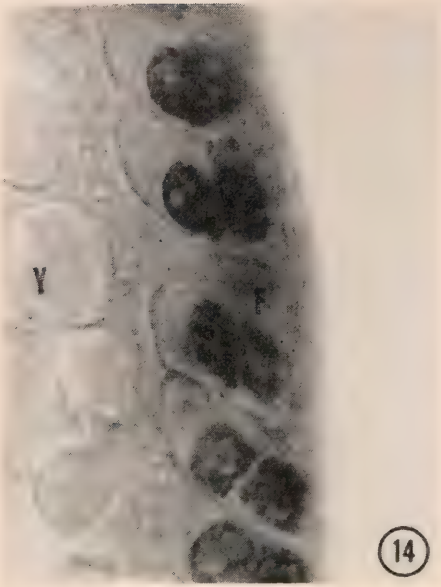
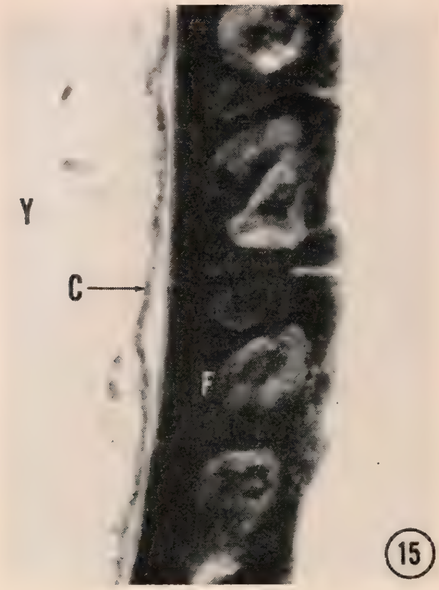
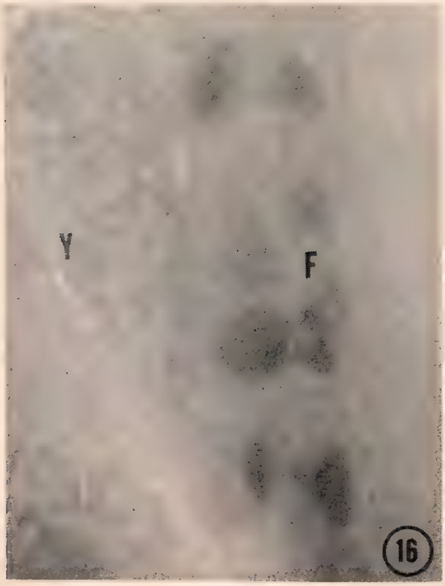
FIGURE 12. Region of prefollicular tissue (P) surrounding young oocytes (O). Note the anaphase (arrow) in the prefollicular tissue. Heidenhain's hematoxylin. Approximately 800 ×.

FIGURE 13. Columnar stage of follicular epithelium (F) surrounding yolk (Y) of oocyte. Each follicle cell is binucleate. Heidenhain's hematoxylin. Approximately 675 ×.

PLATE V







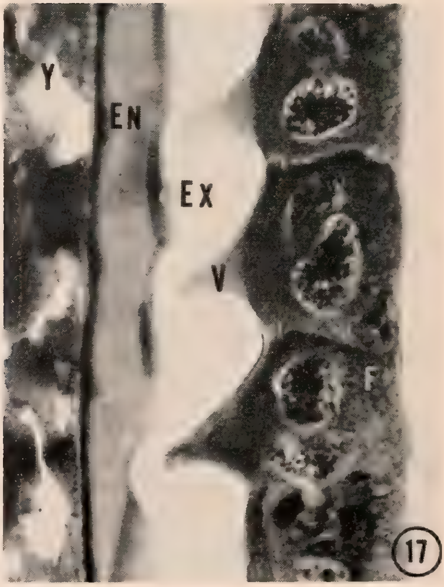


FIGURE 14. Cuboidal stage of follicular epithelium (F) with Feulgen staining. All of the nuclei of the follicle cells are Feulgen-positive. (Y) yolk. Approximately 850 ×.

FIGURE 15. Follicle cells (F) and yolk (Y) of oocyte, showing basophilia in cytoplasm, in nuclear granules of the follicle cells, and in the cortical cytoplasm (C) of the yolk. Azure B bromide. Approximately 850 ×.

epithelium, and the process of chorion deposition in *Rhodnius* have been described in detail by Beament (1946).

B. Autoradiographic results

1. DNA. The incorporation of tritiated thymidine was restricted primarily to nuclei. After 15 minutes of thymidine uptake, it was possible to detect granules in the emulsion above the nuclei of all types of ovarian tissue. Trophocyte nuclei (Fig. 18) in all portions of the germarium, as well as migratory nuclei, were observed to be engaged in thymidine incorporation. The follicular tissue in its various stages, from the prefollicular region to the cuboidal follicular region in the process of secreting the exochorion, showed a higher rate of thymidine incorporation than any other tissue. The tight packing of the young follicle cells resulted in a high density of nuclei per tissue section, thus making it difficult to localize individual clumps of granules above specific nuclei (Fig. 19). However, general labeling was found above the nuclear region of the cells, and there was little if any cytoplasmic labeling in this region. In addition, there was no indication of thymidine incorporation within the nutritive cords, or of the early passage of labeled material down the cords (Fig. 19). Some granules were apparent above the cytoplasmic region of the older follicle cells. The great preponderance of incorporation, however, was nuclear (Fig. 20). No granules were observed above the germinal vesicle of any stage. In addition to ovarian labeling, incorporation was observed in the nuclei of the fat body cells (Fig. 21) and the midgut cells.

This process of predominant nuclear incorporation of thymidine into DNA and retention of the DNA by the nuclei continued for up to 4 to 6 hours. At this time it became possible to detect the appearance of granules above the cytoplasm of the ovarian cells. The spread of radioactivity continued gradually, after 6 to 8 hours extending over the yolk. Unfortunately, under the conditions of the experiment, the time sequence of the label migration did not show a clear progression from the nuclei to the cytoplasm and then to the yolk *via* the nutritive cords. There was instead a gradual leakage which occurred in all regions of the ovary and was rather difficult to trace.

2. RNA. The pattern of incorporation of labeled uridine into RNA showed strong nuclear labeling in the trophocytes (Fig. 22) and in the follicle cells (Fig. 23). The three zones of the apical trophic tissue contained labeled cells, but apparently incorporation occurred in an asynchronous manner, for some cells gave no evidence of uridine uptake. This was in contrast to the situation in the follicle cells, where all nuclei were found to be heavily labeled in all regions from the prefollicular zone down to the follicular epithelial cells just before the process of exochorion deposition.

Ovaries fixed 30 minutes after administration of the uridine showed no label in the mature follicle cells which had completed exochorion formation. When

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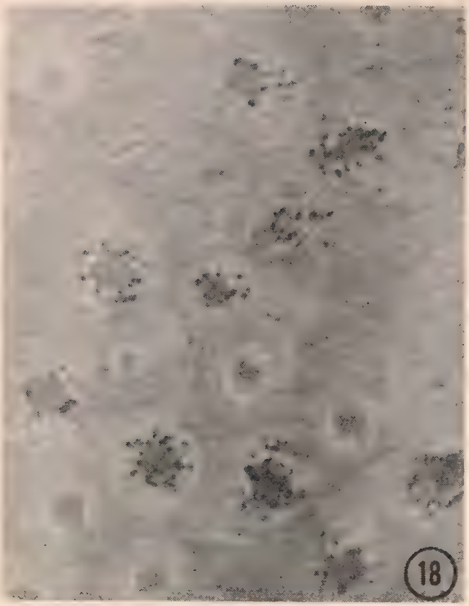
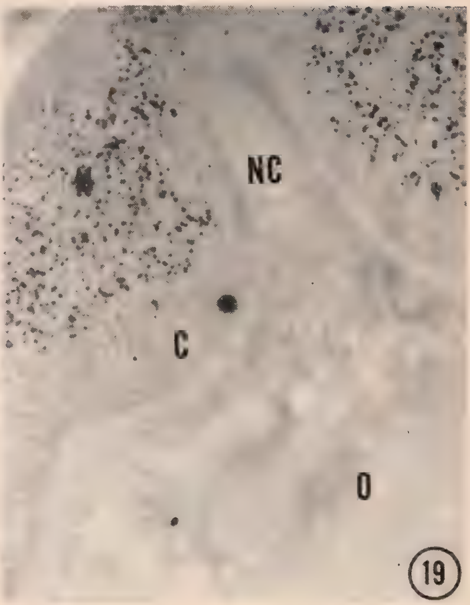
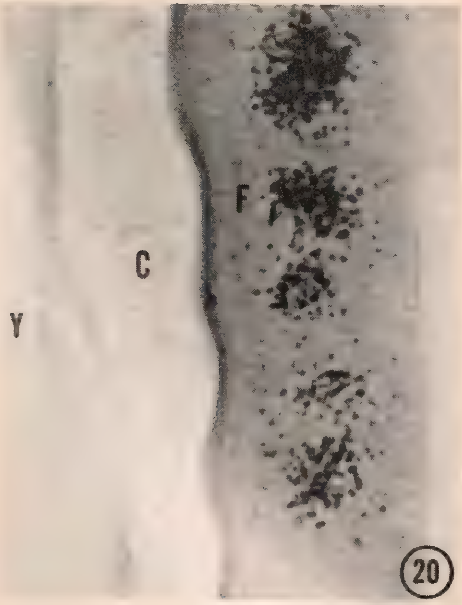
FIGURE 16. Section adjacent to the one shown in Figure 15, demonstrating the loss of basophilia after two hours of ribonuclease incubation. (F) follicular epithelium; (Y) yolk. Azure B bromide. Approximately 850 ×.

FIGURE 17. Follicle cells (F) in process of imprinting the surface sculpturing on the chorion of the mature oocyte. (V) villus of follicle cell; (EX) exochorion; (EN) endochorion; (Y) yolk. Heidenhain's hematoxylin. Approximately 850 ×.

PLATE VI







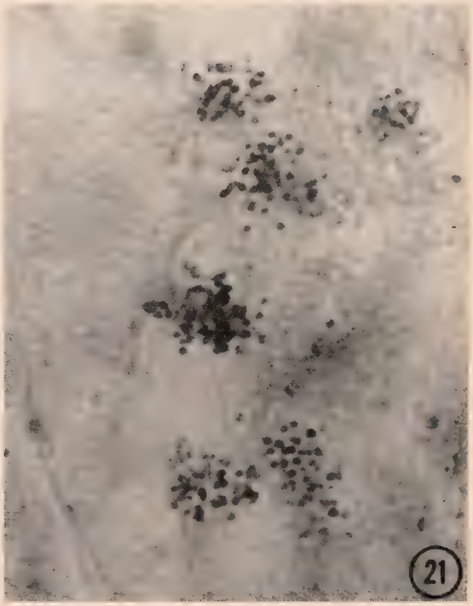


FIGURE 18. Granules above nuclei of trophocytes. Tissue fixed one hour after injection of H^3-thymidine. Approximately 1050 ×.

FIGURE 19. Granules above tightly packed nuclei (N) of young follicular epithelium. No granules are present, at this time, above the cytoplasm (C) of the follicle cells, above the

the 30-minute "pulse" of labeled uridine was followed, the anteriormost of these exochorion-follicle cells gave evidence of labeling in 4 to 6 hours, indicating the rate of movement of the cells and of the oocyte with which they were associated. At some time from 16 to 48 hours, the movement of the follicle cells had progressed to the point where all of the mature follicle cells were labeled.

In following the intracellular movement of label, there was indicated after one to two hours a gradual drift-out from all nuclei (Figs. 24 and 25) into the cytoplasm. This movement continued over a period of time until after 12 hours the cytoplasm was uniformly labeled. There was also a transfer of some of the label to the yolk during this period. The same sequence of uridine incorporation and RNA movement occurred in the fat body and in the midgut, though the midgut rate of incorporation was considerably lower. No incorporation was noted in the germinal vesicle.

3. Protein. The study of protein synthesis, as indicated by the early incorporation of labeled leucine, showed an even distribution of granules above the nuclei and cytoplasm of the germarium (Fig. 26). A much heavier granulation was found above all stages of the follicular tissue (Fig. 27). In addition, the emulsion above the nuclei and cytoplasm of the fat body and midgut contained evenly distributed grains of reduced silver. No granules were noted above the germinal vesicle at any stage.

After two hours it was possible to detect a transfer of some of the label in the follicle cells to the oocyte yolk (Fig. 28). This process continued over a period of several hours. The proteinaceous components of the chorion were found to be deposited in discrete layers, as indicated by Beament (1946), though the autoradiographic timing methods used were not precise enough to distinguish all of the seven chorion layers that Beament described. Figure 29 shows heavy granulation above the layer which is here interpreted as the soft exochorion layer of Beament.

Discussion

A. DNA

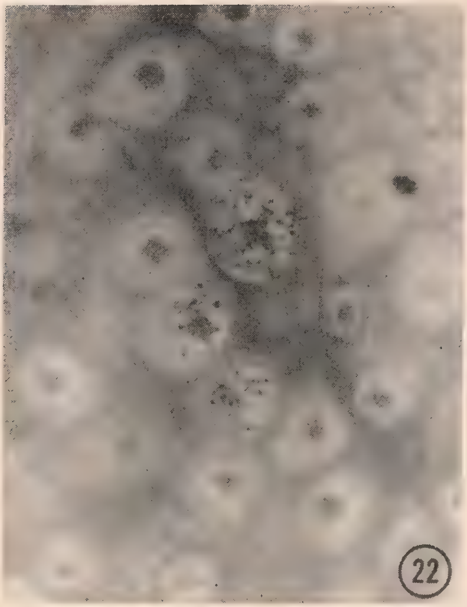
The evidence indicates that the trophic tissues of the *Rhodnius* ovary are active in transferring DNA in a partially depolymerized form to the growing oocyte. The breakdown of migratory nuclei in the germarium releases Feulgen-positive material into the trophic core, yet no droplets can be found in the strands leading to the oocytes. Processes similar to this have been reported by Schrader and Leuchtenberger (1952) for the coreid bug, *Acanthocephala*, and by Bonhag (1955) for the milkweed bug, *Oncopeltus*. It is conceivable that the failure to detect DNA in the nutritive cords and in the oocyte cytoplasm by use of the Feulgen technique is a result of the great dilution that the DNA would undergo when released. It would seem more reasonable, however, that the DNA is depolymerized to some Feulgen-negative form before being transferred to the oocyte.

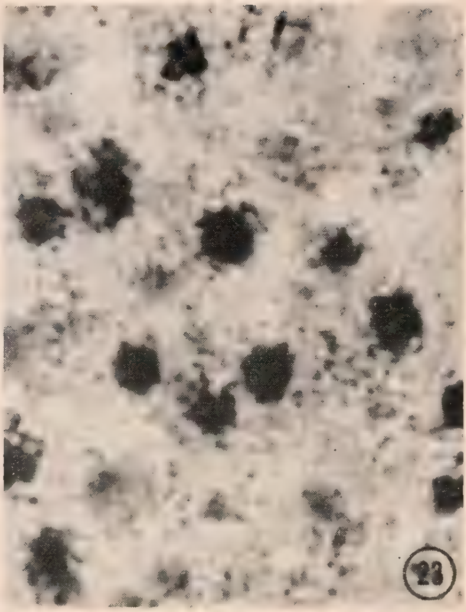
nutritive cord (NC), or above the oocyte (O). Tissue fixed one hour after injection of H^3-thymidine. Approximately 650 ×.

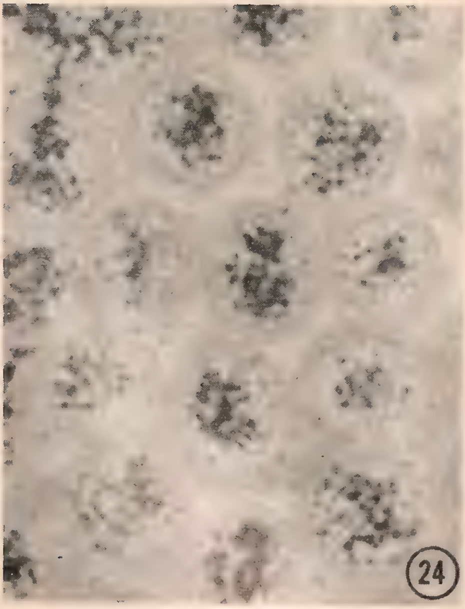
FIGURE 20. Granules predominantly above nuclei of follicular epithelium (F). No granules are present above the chorium (C), or above the yolk (Y). Tissue fixed one hour after injection of H^3-thymidine. Approximately 1250 ×.

FIGURE 21. Granules above nuclei of fat body cells. Tissue fixed one hour after injection of H^3-thymidine. Approximately 1500 ×.

PLATE VII







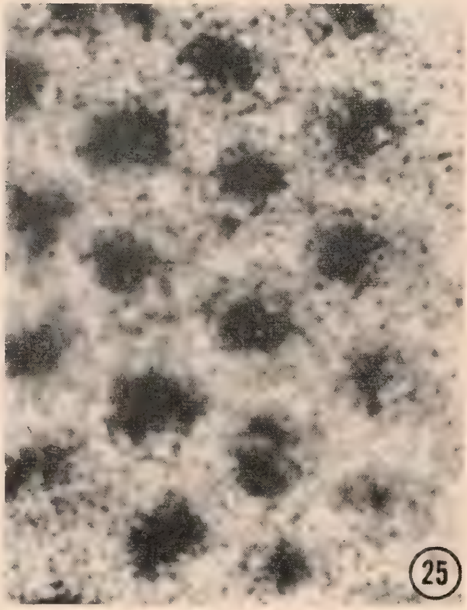


FIGURE 22. Granules above nuclei of trophocytes. Thirty-minute uptake of H^3-uridine allowed before radioactive precursor was diluted out. Tissue fixed one hour after H^3-uridine injection. Approximately 1050 ×.

FIGURE 23. Granules predominantly above nuclei of trophocytes, with some granulation above cytoplasm. Thirty-minute uptake of H^3-uridine allowed before radioactive precursor was diluted out. Tissue fixed two hours after H^3-uridine injection. Approximately 1050 ×.

The autoradiographic results tend to support this conclusion. The nonspecific spread of granules through the cytoplasm of the germarium, when the time course of the tritiated thymidine pulse is studied, suggests the passage of a diffusible form of DNA from nuclei to cytoplasm and eventually into the cytoplasm of the growing oocyte. The autoradiographs indicate that this process also takes place in the follicle cells. At the same time that the label is observed moving from the nuclei of the follicular epithelium to the cytoplasm, the Feulgen test shows that the intact DNA of the follicle cell is retained exclusively in the nuclei. Here again, a depolymerization of DNA into a diffusible Feulgen-negative form is suggested.

Nigon and Nonnenmacher (1961) have reported the incorporation of tritiated thymidine into the cytoplasm as well as the nuclei of nurse cells in the *Drosophila* ovary. However, their flies were studied an hour after administration of the precursor, and it is conceivable that their cytoplasmic label represents migration from the nucleus to the cytoplasm during the one-hour uptake period, rather than the cytoplasmic incorporation that they postulated. Similarly, Durand (1958) has shown that in the ovary of *Gryllus*, the cytoplasm of the mature follicle cells, as well as the basophilic network of the oocyte, show evidence of the presence of a label after the administration of tritiated thymidine. Granules were found above the cytoplasmic villi of the follicle cells, strongly suggesting the migration of the label from the follicle cells to the oocyte. However, no pulse type experiments were performed in either of these studies in an attempt to determine the time sequence of the label incorporation and migration. Consequently, there was no way of distinguishing sites of incorporation from sites of storage after migration. Jacob and Sirlin (1959) have presented autoradiographic evidence for the loss of DNA from the trophic tissue of the *Drosophila* ovary, and the transfer of either DNA or acid-soluble nucleosides to the ooplasm.

Zalokar (1960b) has discussed the theoretical considerations involved in the autoradiographic determination of the site of formation of a substance from its precursor, as opposed to the determination of the intermediary storage site of the synthesized substance. He concluded that if one is interested in localizing the site of formation, that in addition to utilizing a precursor of high specific activity in a high concentration, the time between the administration of the precursor and the immobilization of the product should be as short as possible, never substantially exceeding the time required to synthesize a complete molecule of the product.

In the present experiment, granules indicating the incorporation of tritiated thymidine were detected exclusively above the nuclei of nurse and follicle cells 15 minutes after injection of the thymidine. This was a substantially longer time than that recommended by Zalokar. Nevertheless, on the basis of the timed pulse experiments, it seems reasonable to assume that the thymidine was incorporated in the nuclei, and migrated to the cytoplasm in a form which was depolymerized sufficiently that it gave a Feulgen-negative test. Furthermore, the migrating labeled material was insoluble enough to be retained in the tissues after washing

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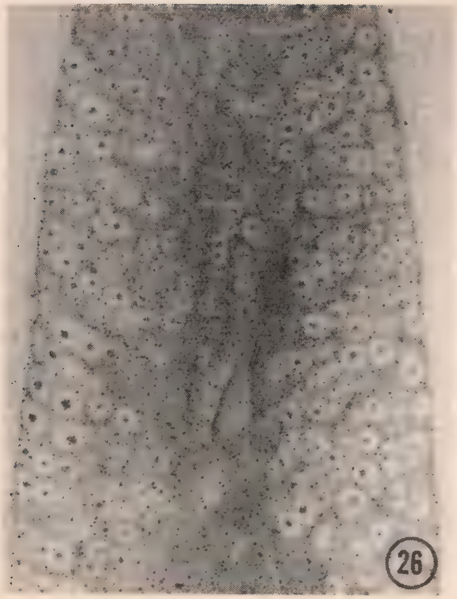
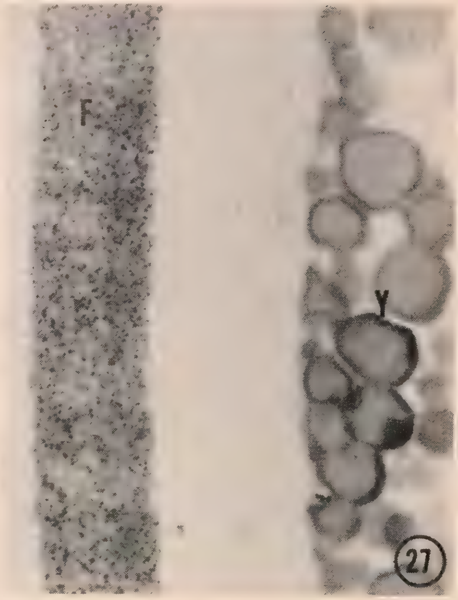
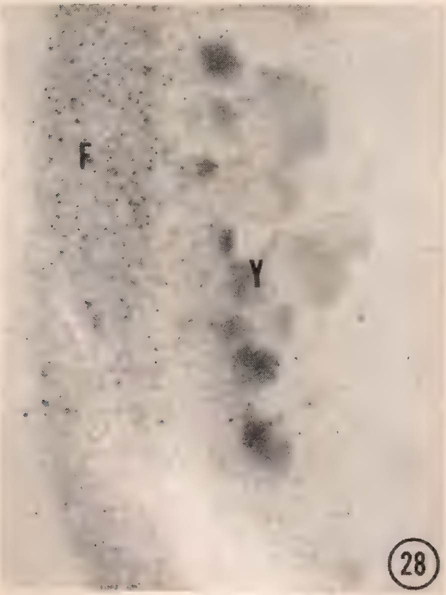
FIGURE 24. Granules above nuclei of follicular epithelium (tangential section). Thirty-minute uptake of H^3-uridine allowed before radioactive precursor was diluted out. Tissue fixed one hour after H^3-uridine injection. Approximately 1100 ×.

FIGURE 25. Granules predominantly above nuclei of follicular epithelium, with some granulation above cytoplasm (tangential section). Thirty-minute uptake of H^3-uridine allowed before radioactive precursor was diluted out. Tissue fixed two hours after H^3-uridine injection. Approximately 1100 ×.

PLATE VIII







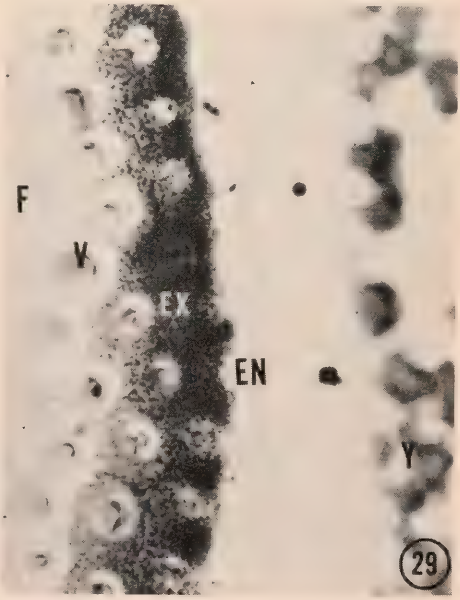


FIGURE 26. Granulation above nuclei and cytoplasm of germarium. Tissue fixed thirty minutes after injection of H^3-leucine. Approximately 300 ×.

FIGURE 27. Granulation above nuclei and cytoplasm of follicular epithelium (F), with no granules above yolk (Y) of oocyte. Tissue fixed thirty minutes after injection of H^3-leucine. Approximately 550 ×.

with reagents as previously described, or was protected from the action of the histological reagents by an association with proteins denatured during fixation.

One is struck by the increase in DNA content in the nuclei of both the apical trophic tissue and the follicular epithelium. In the nurse cells there is a distinct increase in the intensity of the Feulgen staining from the nuclei of the first through the third zones. On the basis of microspectrophotometric measurements of Feulgen staining in the ovary of the bug, *Acanthocephala*, Schrader and Leuchtenberger (1952) concluded that the irregular increase in DNA content of the more distal nuclei could best be explained by a process of fusion. In *Rhodnius*, the incorporation of thymidine in all zones of the germarium makes it apparent that endopolyploidy is an important factor in the increase, though cytological study suggests that fusion of nuclei also occurs.

The uptake of thymidine by the prefollicular tissue and by the young follicular epithelium is probably associated with the active mitosis that occurs in these tissues. However, after the follicle cells become binucleate, no further mitoses are observed. Inasmuch as the nuclei continue to incorporate thymidine and increase in Feulgen staining intensity during this period, it is apparent that endopolyploidy is occurring.

One can only speculate on the significance of the considerable rate of synthesis of DNA by the trophic tissues of the ovary, and the apparent transfer of some of this DNA to the growing oocytes. The phenomenon appears to be widespread in insects. Kaufmann *et al.* (1953) reported that in the ovary of the wasp, *Habrobracon*, polyploid nurse-cell nuclei disintegrate and are engulfed by the egg cytoplasm, with the DNA from the nurse-cells being quickly broken down and dispersed throughout the egg. The authors concluded that DNA building blocks are probably stored in the egg cytoplasm. Durand (1955) has shown that the unfertilized egg of *Gryllus* contains 1600 times more DNA than the spermatozoan. A reserve of DNA or of DNA precursors has been found in the egg of *Drosophila* by Levenbook *et al.* (1955), and by Nigon and Daillie (1958).

It seems unlikely that any genetic information is carried over in the transfer of DNA from the maternal ovary to the developing egg. It would appear more reasonable that the reserve of DNA or of its precursors serves as a readily available source of building blocks to supply the needs of the cleavage nuclei.

All of the excess DNA synthesized in the ovary, however, is not utilized for this egg reserve. The follicle epithelial nuclei of *Rhodnius* continue synthesizing DNA even after the process of chorion deposition over the oocyte has been completed, and any further transfer of material between the follicle cell and the oocyte would be impossible. The apparent overproduction of DNA is a phenomenon which is not unknown in insects. Wigglesworth (1942, 1948) has shown that during the height of cell division in the epidermis of *Rhodnius* and other insects, mitosis is so exuberant that many more cells are produced than will be needed

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FIGURE 28. Granulation above follicular epithelium (F), and above yolk (Y). Thirty-minute uptake of H^3-leucine allowed before radioactive precursor was diluted out. Tissue fixed six hours after injection of H^3-leucine. Approximately 550 ×.

FIGURE 29. Section of mature oocyte cut almost on a tangent. This has the effect of stretching out the horizontal dimension of the section. (F) follicle cells; (V) villus of follicle cell; (EX) exochorion; (EN) endochorion; (Y) yolk. Granulation is heavy above the exochorion. Tissue fixed four hours after injection of H^3-leucine. Approximately 500 ×.

to form the new integument. The unwanted cells die, releasing Feulgen-positive droplets which are apparently assimilated by the neighboring cells.

B. RNA

Clearly, both the trophocytes and the follicular epithelial cells function in the transfer of RNA to the oocyte of *Rhodnius.* The histochemical evidence for the passage of RNA from ovarian trophic tissues of insects to the growing oocytes has been reviewed by Bonhag (1958). The histochemical observations on *Rhodnius* are in accord with the previous findings on the apparent passage of RNA from the trophocytes through the RNA-rich nutritive cords in telotrophic ovaries. The *Rhodnius* ovariole exhibits a reduction in the intensity of oocyte basophilia due to RNA as the oocyte enlarges. Because of the enormous amount of yolk added to the oocyte during growth, it is likely that this reduction in basophilia is caused by a dilution of the RNA rather than by a reduction in it.

The present autoradiographic studies have confirmed the observations on the transfer of RNA from the trophocytes, and extended these findings to the follicle cells. The autoradiographic observations on the intracellular movement of RNA are consistent with the results of King and Burnett (1959), Zalokar (1960a), and Sirlin and Jacob (1960), demonstrating the incorporation of labeled precursors into the RNA of the nurse cell and follicle cell nuclei of *Drosophila,* with the subsequent movement of the label to the cytoplasm.

Inability to demonstrate uridine incorporation in the mature follicle cells which have completed the process of exochorion deposition is of particular interest. On the basis of the hypothetical function of the RNA, this is what might have been expected. One might conceive of the nuclei of the trophic tissue being involved in the synthesis of at least two functionally different classes of RNA. First of all, we might expect the synthesis and transfer of RNA, capable of transmitting genetic information which regulates the formation of oocyte protein by the cytoplasm of the trophic tissue. Presumably, the protein components of the exochorion are the last proteins transferred to the oocyte by the follicle cells before the latter are autolyzed. There would thus be no requirement for further synthesis of this type of informational RNA after the formation of the exochorion.

Second, some of the RNA produced by the trophic tissues is transferred, as has been shown, to the oocyte, where it may serve as a reserve for the developing embryo. The further transfer of this RNA to the oocyte would be impossible after the formation of the chorion.

C. Protein

The study of the 30-minute pulse of incorporation of tritiated leucine clearly indicates the passage of protein from the trophic tissues to the oocyte. Synthesis appeared to take place throughout the cytoplasm and nuclei of all the trophic cells. King and Burnett (1959), studying the uptake of tritiated glycine by *Drosophila* ovaries, also reported amino acid incorporation by both cytoplasm and nuclei. Exclusive incorporation in the cytoplasm of the *Drosophila* ovary during the first minute after injection of tritiated leucine, however, was noted by Zalokar (1960a). After 16 minutes, the nuclei became as heavily labeled as the cytoplasm.

Incorporation of tritiated histidine into the nurse and follicle cells of *Calliphora* was shown to be predominantly cytoplasmic during the first 15 minutes after injection, by Bier (1962). It seems possible that the nuclear label in the present experiment represented migration from the cytoplasm rather than the initial site of protein synthesis. Results similar to those of Zalokar and Bier might have been obtained if the *Rhodnius* ovaries had been fixed a short time after injection, instead of after 30 minutes.

In *Rhodnius*, the greatest increase in oocyte size is observed after the oocyte has been separated from the nutritive cords, and the responsibility for nutrition has been taken over by the follicular epithelium. Because of this, and because of the fact that the number of reduced silver grains is considerably greater in the emulsion above the follicle cells than above the trophocytes, it is obvious that the follicular epithelium plays a more important role in the transfer of proteins to the oocyte.

The large number of reduced silver grains above the fat body indicates that it was almost as active in protein synthesis as the follicular epithelium, the two tissues showing considerably more amino acid incorporation than any other tissues studied. It is probable that much of the protein synthesized by the fat body is transferred to the blood and plays a role in yolk formation. Shigematsu (1958) has reported the *in vitro* incorporation of labeled amino acids into the larval fat body of the silkworm, *Bombyx*, with the subsequent release of synthesized protein into the incubating medium. Wigglesworth (1943) has suggested that much of the normal protein in the yolk may be synthesized elsewhere and merely transferred to the egg by the follicle cells. Evidence for this process taking place in the silkworm, *Samia*, has been obtained by Laufer (1960a, and 1960b) by means of zone electrophoresis of tissue proteins in starch. A female-specific protein is present in high concentration in the fat body, which seems to be the source of this protein. Laufer has been able to demonstrate the appearance of the protein in the blood, and later in the eggs. This is consistent with the immunochemical observations of Telfer (1954, 1960), who demonstrated that a number of antigens present in the blood of the Cecropia moth were accumulated by the oocytes. He also found that several oocyte antigens could not be detected in the blood. These latter antigens may have represented proteins that were synthesized in the ovary.

There is thus evidence from various sources that the synthesis of oocyte protein may be both ovarian and extra-ovarian. That both processes may take place in *Calliphora* has been shown autoradiographically by Bier (1962). Ovarian synthesis of protein is detectable one to 15 minutes after injection of tritiated histidine. Forty minutes after injection of the precursor, a protein, apparently synthesized outside the ovary, accumulates at the periphery of the ooplasm and migrates into the oocyte in the form of rounded platelets. No clear distinction between these two processes was observed in the present study. The transfer of label from the follicular epithelium to the interior of the oocyte was instead a slow and uniform process, with no indication of either the accumulation of protein at the periphery of the oocyte, or of the inward migration in the form of platelets. There is some circumstantial evidence, however, that suggests that much of the protein synthesized by the *Rhodinus* fat body may be utilized by the ovaries.

Allatectomized bugs, having little oocyte development, show only negligible amino acid uptake by the fat body. On the other hand, control bugs, with full ovarian development, reveal that the fat body is active in amino acid incorporation. It is probable that the transfer of label from the follicle cells to the oocytes in the present study represents a summation of both processes: follicular epithelial synthesis, and extra-ovarian synthesis.

Bonhag (1958) has reviewed the literature on the formation of yolk protein in insect ovaries. At one time or another, practically every cellular structure known to the light microscopist has been reported to produce or to transform into yolk protein. These conclusions have generally been based on cytological evidence so tenuous as to be of limited value. It is quite evident from the present studies that in *Rhodnius,* at least, most of the yolk protein is transferred to the oocyte from the follicle cells. How much of the protein is actually synthesized by the follicular epithelium, and how much of it is merely transferred to the epithelium from the blood is difficult to determine. Contrary to many of the previous reports on vitellogenesis, as cited by Bonhag, the germinal vesicle appears to play only a minor role in the synthesis of protein.

SUMMARY

1. Histochemical and autoradiographic techniques were applied to *Rhodinus prolixus* during vitellogenesis.

2. Examination of the ovary, fat body, and midgut demonstrated that DNA was synthesized exclusively in the nuclei of these tissues. There appeared to be a transfer of some of the DNA in a partially depolymerized form from the trophic tissues of the ovary to the growing oocyte.

3. RNA was synthesized in the nuclei of the ovary, fat body, and midgut, and subsequently was transferred to the cytoplasm of these tissues. Some of the newly synthesized RNA passed from the trophic tissues of the ovary to the enlarging oocyte.

4. Protein was found to be synthesized most actively in the follicular epithelial tissues of the ovary, and in the fat body. Evidence of protein synthesis was also found in the other regions of the ovary, and in the midgut. The passage of newly synthesized protein from the follicular epithelium into the oocyte was noted. Synthesis of yolk protein by the oocyte itself appeared to be negligible.

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THE ROLE OF THE GONADOTROPIC HORMONE IN THE SYNTHESIS OF PROTEIN AND RNA IN RHODNIUS PROLIXUS (HEMIPTERA)

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There is considerable evidence that the corpus allatum is involved in ovarian activity in a wide variety of insects. Since Wigglesworth (1936) demonstrated the gonadotropic action of the corpus allatum in *Rhodnius prolixus*, the presence of the gland has been found to be necessary for deposition of yolk in the eggs of species representing the Orthoptera, Blattaria, Hemiptera, Dermaptera, Lepidoptera, Diptera, and Coleoptera (Johansson, 1958). In some insects, such as *Calliphora* (Thomsen, 1952) and *Schistocerca* (Hill, 1962), it appears that a gonadotropic action is exercised by the neurosecretory cells of the brain. In view of the physiological and chemical similarities of the secretions of the corpus allatum and of the neurosecretory cells (Gilbert and Schneiderman, 1959), this is not too surprising.

Gilbert and Schneiderman (1961) have reviewed the evidence that strongly suggests that the corpora allata are intimately involved in various phases of protein metabolism. The present study is concerned with testing the relationship between the gonadotropic action of the corpus allatum and protein synthesis, by studying the incorporation of labeled precursors into DNA, RNA, and protein by means of the autoradiographic technique in *Rhodnius*.

MATERIAL AND METHODS

Female *Rhodnius* adults, which had emerged three to four days previously, were used in the experiments. The bugs were transilluminated in a darkened room to make certain that no well developed oocytes were present. This examination enables one to detect females with well developed ovaries by observing the pink color imparted to the developed oocytes by the hemoglobin breakdown products deposited in the yolk (Wigglesworth, 1936). Females with undeveloped ovaries were given a blood meal and were decapitated 24 hours later.

Decapitation was carried out as follows. A thin layer of a mixture of 60% beeswax and 40% rosin was deposited around the head and the anterior portion of the thorax. This was applied as a molten drop with a loop of nichrome wire electrically heated to just above the melting point of the wax mixture. Decapitation was then accomplished by ligating the head with a fine thread either just anterior or just posterior to the corpus allatum. The relationship between the external morphology of the head and the localization of the corpus allatum in

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Rhodnius has been indicated by Wigglesworth (1936). Wounds were sealed with a drop of the beeswax-rosin mixture applied as described above. The decapitated bugs were maintained at 28° C. in a high-humidity rearing chamber for periods ranging from two to six weeks.

The operated bugs were studied histologically, histochemically and autoradiographically at varying times after decapitation. The techniques have been described previously (Vanderberg, 1963).

RESULTS

Bugs which were decapitated with removal of the corpus allatum did not complete development of ripe oocytes. On the other hand, control bugs which were decapitated, but which retained the corpus allatum, typically developed 20 to 30 ripe oocytes (Fig. 1).

As Wigglesworth (1936) first showed, in bugs deprived of the corpus allatum, egg development continues normally until the stage at which the oocyte loses its connection with the trophocyte cells. At this point, when the oocyte is about 0.45 mm. in length, its development is arrested and it dies. The follicular cells, instead of producing yolk, seem to proliferate amitotically and absorb the dead oocyte (Fig. 2).

The operated controls presented the same pattern of incorporation and transfer of label as the normal unoperated bugs (Vanderberg, 1963). Bugs which had had the corpus allatum removed, however, showed marked differences in the way in which two of the labeled precursors were incorporated.

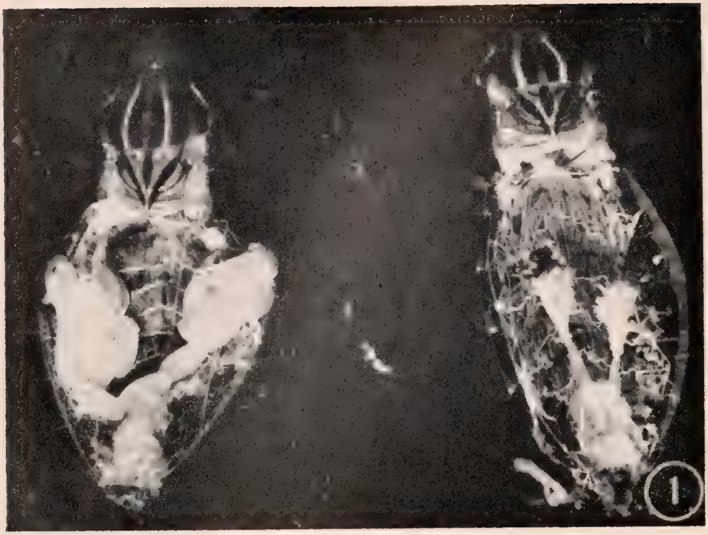
Thymidine incorporation into DNA in the allatectomized animals appeared to take place in the same manner as it occurred in the normal animals and in the operated controls. Of course, the ovaries of the allatectomized animals had no well developed follicles to study. The early stages of oocyte development, however, indicated approximately the same rate of incorporation of tritiated thymidine as the normal ovary.

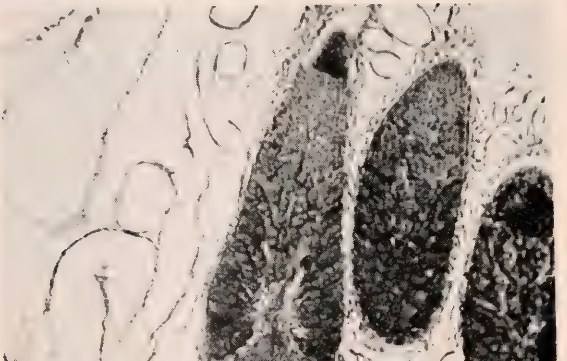
The allatectomized bugs showed a drastic inhibition of the incorporation of uridine into RNA and of leucine into all tissues studied. The label, as determined by autoradiography, was detected in the ovary, fat body, and midgut. However, the number of silver granules was considerably reduced in the emulsion above tissues from allatectomized specimens in comparison to tissues from normal and operated control animals.

DISCUSSION

The present study has established an experimental correlation between the presence of the corpus allatum and the active synthesis of protein and RNA in the *Rhodnius* ovary, fat body, and midgut. Apparently, the fact that corpus allatum activity is not necessarily correlated with DNA synthesis in these tissues indicates that DNA synthesis may be dissociated from the synthesis of protein and RNA.

A relationship between gonadotropic hormone and protein synthesis in the desert locust, *Schistocerca*, has been demonstrated by Hill (1962). An "active" neurosecretory system resulted in developing ovaries and a high concentration of hemolymph protein. Cauterization of the gonadotropic neurosecretory cells





FIGURES 1–2.

produced a lowering of the hemolymph protein concentration and an elevation of the concentration of free amino acids.

The hormonal control of a specific protein, proteinase, has been reported by Thomsen and Møller (1959). The gonadotropic neurosecretory granules in *Calliphora* are carried *via* the *nervus oesophagei* to the gut, where they stimulate proteinase activity. In addition, Dadd (1961) has shown that in *Tenebrio* the proteinase activity of the midgut seems to be controlled by humoral factors. It might be suggested that in such cases the synthesis of protein yolk would be limited simply by the availability of amino acids derived from digestion in the gut. This, however, appears unlikely, since there is some evidence which shows that protein synthesis may be inhibited even in the presence of available amino acids. Thus, there is a very distinct increase in free amino acids after removal of the corpora allata in *Dixippus* (L'Hélias, 1953). Also, an accumulation of free amino acids in the hemolymph of *Schistocerca* was noted by Hill (1962) after destruction of the neurosecretory cells which produce the gonadotropic hormone.

Possibly, the action of the gonadotropic hormone on protein or RNA synthesis is a direct one. However, it is also conceivable that the action is indirect. The stimulation of oxygen consumption caused by the corpus allatum has been demonstrated in the intact insect (Thomsen, 1949; DeWilde and Stegwee, 1958; Sägesser, 1960; Novák and Sláma, 1962), in tissue homogenates (DeWilde and Stegwee, 1958), and in isolated mitochondria (Clarke and Baldwin, 1960; Stegwee, 1960). In addition, Stegwee (1963) has demonstrated by means of electron micrographs that the corpora allata have a stimulatory effect on mitochondrial morphology in the intact *Leptinotarsa* adult. If an action on oxidative metabolism happened to be the primary effect of the gonadotropic hormone, it would tend to control protein synthesis indirectly by controlling, perhaps, the availability of adenosine triphosphate necessary for protein synthesis.

Gilbert and Schneiderman (1961) have cited the widespread evidence that establishes the importance of the corpora allata in protein metabolism. The precise role that the corpora allata play, however, is still in doubt. A number of metabolic activities appear to be concurrently induced by the gonadotropic hormone of the adult insect. These activities include protein synthesis, RNA synthesis, oxygen consumption, gut proteinase activity, muscle transaminase activity (Wang and Dixon, 1960), and the stimulatory effect on the morphology of the mitochondria. Any one of these activities, if it happened to be the primary target of the hormone, would be capable of influencing the other metabolic activities by some sort of feedback mechanism, or directly by substrate or energy limitation.

The work of Becker (1962) suggests that the juvenile hormone in *Drosophila* may function by directly activating the gene. The striking similarity, and perhaps identity, of the juvenile hormone and the gonadotropic hormone is well known

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FIGURE 1. *Rhodnius* adults dissected three weeks after surgical treatment. Bug on the left was decapitated, but retained the corpus allatum. Normal ovarian development with production of ripe oocytes resulted. Bug on the right was decapitated with removal of the corpus allatum. There was little ovarian development, but instead an enlargement of the fat body.

FIGURE 2. Sagittal section of ovariole of bug three weeks after allatectomy. The initial stages of oocyte development proceed normally, but the follicle cells have absorbed the enlarged oocyte to form an empty follicle.

(Wigglesworth, 1954, 1961). In view of this, the possibility that the gonadotropic hormone may act directly at the level of the gene is an attractive one. There would seem to be insufficient data at this time to establish any primary mode of action.

SUMMARY

1. The hormone of the corpus allatum in *Rhodnius prolixus,* as well as in many other insects, has been found to be necessary for the deposition of yolk to occur in the growing oocytes of the ovary. Experiments were conducted with *Rhodnius* to test the hypothesis that this gonadotropic action is exercised by controlling protein synthesis in the insect.

2. Autoradiographic studies of the incorporation of tritium-labeled precursors into DNA, RNA, and protein were undertaken on decapitated bugs that had the corpus allatum removed, and on decapitated controls that retained the gland.

3. The hormone appeared to have little or no effect on DNA synthesis, but allatectomized bugs showed a drastic inhibition of RNA and protein synthesis in all tissues studied.

4. It is not clear whether the hormone has a direct or an indirect action on protein synthesis.

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RADIALLY ORIENTED CLEAVAGE IN BLASTULAE AND IN GASTRULAE OF THE STARFISH, ASTERIAS FORBESI

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Orientation of the mitotic spindle and of the cytoplasmic cleavage plane are generally of greater interest to the plant histologist than to those working with animal tissues. Although the earlier zygotic divisions have been studied at length, only an occasional investigator has considered patterns of division in connection with later developmental events (*e.g.*, Pohley, 1959; Lipp, 1959). Highly oriented divisions seem particularly likely to occur during the formation of starfish blastulae and gastrulae: the arrangement of cells in just one layer could be explained, at the first level, by radial cleavage planes. This aspect of development seems to have received less attention than either the earlier or the later phenomena.

Embryologists of the late nineteenth and of the early twentieth centuries discussed in great detail the arrangements of cleavages up to the 16- and to the 32-cell stages. They frequently used such terms as "meridional" and "equatorial" to describe the division planes (*e.g.*, Seeliger, 1892; Boveri, 1901). Zeigler (1924) gave a particularly precise account, for several echinoderms, of the "vertical" and of the "horizontal" cleavages which bring the zygote to 8, to 16, and to 32 cells. Korschelt (1936) attributed the several basic patterns (radial, spiral, and bilateral) to cleavage plane direction. He too used "meridional" and "equatorial" for divisions up to the 32-cell stage. Obviously, the terms "meridional," "equatorial," "vertical," and "horizontal" imply divisions which cut the zygote in the same way that radial cleavages cut the blastula.

Metschnikoff (1885: see his Figs. 57–63) pictured blastulae with radially dividing blastomeres, and described the cleavages as being radial (his p. 664). He said nothing about maintaining a single layer of cells, but his figures show this characteristic. In a discussion of the coeloblastula, Korschelt (1936) mentioned radial cleavage (p. 93), one-layered wall (p. 94), and the production of the several-layered condition by tangential cleavages (p. 95); however, he did little more than remark on these conditions in passing.

Except for a photomicrograph in Immer's (1957) paper on cytochemical aspects, the only recent reference I have found is that of Wolpert and Gustafson (1961) on sea urchin blastulation. Observations on living embryos led these authors to stress the importance of radial cleavages in maintaining the single layer of cells. They considered this restricted orientation (p. 381) ". . . an essential feature in blastula formation."

My observations were made on fixed, sectioned, and stained blastulae and gastrulae. Numerous mitotic figures indicated that radial cleavage is the rule, not only through blastulation but during gastrulation as well. Additional phenomena of interest, found in this same material, include occasional cleavages passing along the longer axis of the cell, and additional patterns of division figure orientation.

MATERIALS AND METHODS

Dr. Evelyn Rosenberg, New York University Medical School, supplied the several collections of *Asterias forbesi* used in this study. I am very happy to acknowledge Dr. Rosenberg's generosity and consideration over the past several summers.

The embryos were fixed in 10% formalin. They were pipetted into the emptied pupal sacs of ants, and the open end of each sac was tied shut with a hair. This kept the embryos together during the subsequent processing, which included dehydration by the tertiary butyl alcohol method (Johansen, 1940) and embedding in Tissuemat, melting point ca. 55° C. Sections were cut at 5, at 7, or at 9 micra. Different thicknesses had specific advantages and disadvantages: thinner sections gave clearer pictures, both visual and on film; thicker sections simplified the interpretations of doubtful orientations and included a greater number of whole mitotic figures per single section.

A number of staining techniques produced excellent preparations: Heidenhain's iron hematoxylin, Capinpin's brazilin (both given in Johansen, 1940), Einarson's gallocyanin (given by Terner and Clark, 1960), and azure A-Schiff. The latter was used as suggested by Himes and Moriber (1956) with 10 minutes' hydrolysis in 1 N HCl at 60° C. This stained practically nothing but the chromosomes. The other techniques showed the spindles clearly and also stained the cytoplasm.

Photomicrographs were taken with a Leitz Wetzlar "Ortholux" microscope equipped with a Leitz Wetzlar "Orthomat" camera attachment.

OBSERVATIONS

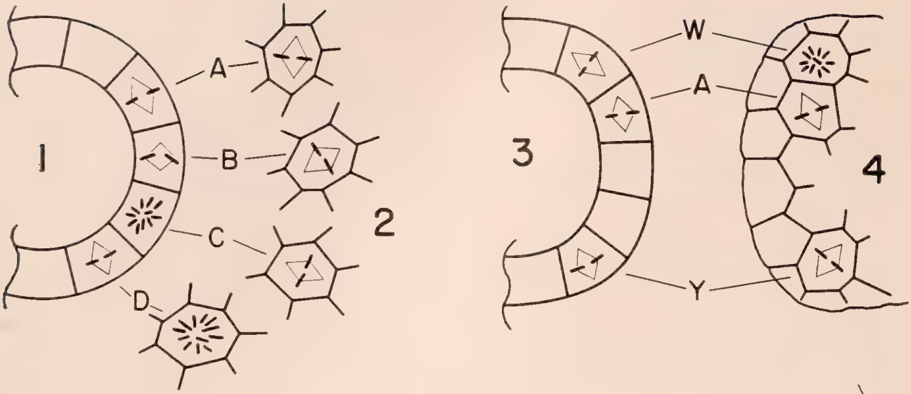
Interpretation of mitotic figure orientation

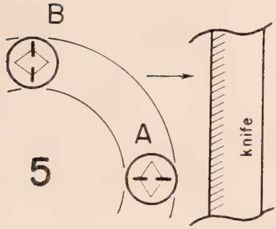
A thin section may include any level of the sub-spherical blastula, so it is difficult at times to interpret the mitotic spindle position in relation to the blastula as a whole. This is of paramount importance, however, since potential radial cleavages may be inferred only from certain alignments of the spindle and of the chromosome clusters.

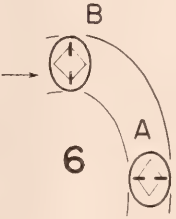
A median section of the blastula appears as a ring of cells. Within these cells, the spindles may lie in various positions which are all consistent with division along the radial plane. In Figure 1, cell *A* shows the spindle in equatorial view; in cell *B*, it is in oblique view; in cell *C*, it is in polar view. All three have a tangentially oriented spindle axis leading to radial cleavage. In contrast, a radially oriented spindle axis with tangentially cleaving cytoplasm and the consequent production of two layers of cells would require the alignment shown in cell *D*. Figures 10–18 are photomicrographs of the first three arrangements.

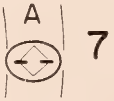
Figure 2 shows cells *A*, *B*, *C*, and *D* of Figure 1 as they would appear in face view on one side (tangential section) of a blastula (see Figs. 19–24). The difference between an orientation leading to radial cleavage (cells *A*, *B*, *C*) and that leading to a tangential cleavage (cell *D*) is clear in these cases, but the two types are not always so easily distinguished.

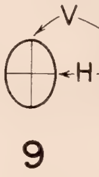
Since serial sections contain slices ranging from a true median section, through

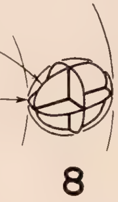












Figures 1–4. Interpretation of spindle orientations relative to radial and to tangential cleavages. Diagrammatic. Figure 1 represents the mid-section of a blastula. The spindle positions of cells *A, B,* and *C* lead to radial division; they appear, respectively, in equatorial view, in oblique view (between equatorial and polar), and in polar view. In cell *D,* the spindle lies in radial orientation which would lead to tangential cleavage. Figure 2 shows the same spindles as they would appear in face view on a tangential section (one side) of the blastula. Figures 3 and 4 show the possible misinterpretation of spindle orientation if one side of the blastula is somewhat flattened due to pressure of an adjacent embryo. The spindle in cell *W* is really aligned for radial cleavage (Fig. 3) but would be misinterpreted as producing a tangential division (Fig. 4) because of the acute curve of the blastula at the edge of the section. Similarly, the spindle of cell *Y* would be misinterpreted as producing a radial cleavage (Fig. 4) whereas it is really aligned to accomplish tangential cleavage (Fig. 3).

Figure 5. Two cells, *A* and *B,* assumed to be spherical, before sectioning. The arrows indicate the direction the paraffin block moves as it passes the knife.

Figure 6. The same two cells showing compression due to sectioning. Cell *B* would appear to be cleaving along its longer axis, but this is an artifact resulting from compression (compare with Fig. 5). Cell *A* would be similarly distorted but would seem to be cleaving along its shorter axis.

Figure 7. If, despite sectioning, a cell in the same position as *A* still has a longer radial diameter and is cleaving radially, it may be interpreted as cleaving lengthwise (see text).

Figure 8. A blastomere cut along the three planes of sectioning, which pass through the three cell axes. The three axes are assumed to be unequal, so that each plane has a longer and a shorter diameter (see text).

Figure 9. The blastomere of Figure 8, shown in face view as it would appear on a tangential section (*e.g.,* Fig. 4) of a blastula. The difference in length between the two visible axes is clear; the third axis lies perpendicular to the page (see text).

cells closer and closer to the sides, and finally the actual side (tangential section) of the embryo, and since the blastula may be deformed by pressure of adjacent embryos, some orientations require careful interpretation. The spindle may seem to be aligned radially but actually lie along the expected tangent. Figure 3 shows a blastula somewhat flattened on one side (due, presumably, to pressure of an adjacent embryo). At cell *W*, the wall is curved abruptly so that this particular cell is likely to be included in a side (tangential) section. Although its spindle is oriented for a radial cleavage, as is clear from Figure 3, it would present an almost polar view on a tangential section (Fig. 4) and in consequence be identified incorrectly as a tangential cleavage. Figures 25 and 26 show this condition in actual cells.

In a similar fashion, it is possible to misinterpret a radially aligned spindle (tangential cleavage) as lying in the tangential direction: *e.g.*, the spindle in cell *Y*, Figure 3, would seem to be so oriented on a tangential section (Fig. 4).

The cases just described are more easily interpreted on thicker sections. Those found on thinner sections, however, can usually be worked out by study of the preceding and of the succeeding serial sections.

Radial cleavage (*tangential spindle orientation*)

With few exceptions, the hundreds of mitotic figures observed were definitely identified as leading to radial cleavage of the blastomeres, relative to the blastular sphere. Interpretation of some spindles was uncertain. Such cases might constitute real exceptions, perhaps associated with other developmental events such as mesoderm formation. On the other hand, they could be divisions in the expected radial plane, but occurring in cells whose positions were peculiarly altered during processing or sectioning.

Although mitosis is no longer synchronous in the blastula stages studied, it is not unusual to find clusters of mitotic cells on the same embryo. Single medial sections often include arcs of two or more dividing cells, all lying in equatorial or in other aspects (Figs. 10–14). Tangential sections (one side of the embryo) may include from several to as many as ten or even more mitotic figures, oriented for radial cleavage (Figs. 19–24). On some tangential sections, mitotic figure positions, *relative to each other*, suggest yet another level of patterning in addition to that already described (Figs. 20, 21, 23). Arrays of this type will be discussed later.

The starfish gastrula also consists of layers one cell thick; therefore, a spindle orientation comparable to that of the blastula may be expected. Since the concept of radial cleavage loses meaning as invagination and gastrocoel formation proceed, comparable evidence for this type of division is found in cleavages perpendicular to the sheet of cells, *i.e.*, perpendicular to a tangent to the curved sheet at that point. Such divisions will maintain a single-layered ectoderm and a single-layered endoderm. Problems relating to spindle figure orientation found throughout sections of gastrulae are much like those encountered in the blastula studies. In these later embryos, however, such factors as the more complex form of the gastrula and the reduced cell size increase the difficulty of interpretation.

Observations on later gastrulae are further complicated by the temporarily changed location of the cleaving cell. It rounds up toward the surface of the cell layer, shifting toward the outer surface of the embryo in the case of the ectoderm, and toward the gastrocoel in the case of the endoderm (Figs. 15–18). The spindle and chromosomes are similarly displaced, so that on tangential sections (sections including one side of the gastrula) the mitotic figure is not in focus

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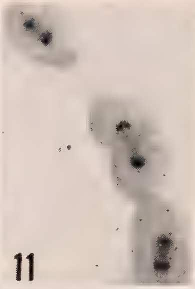
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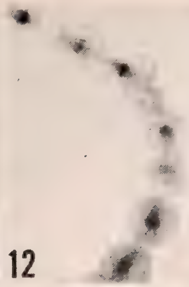
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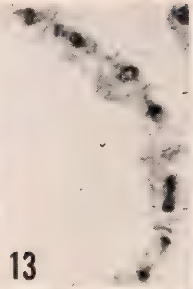
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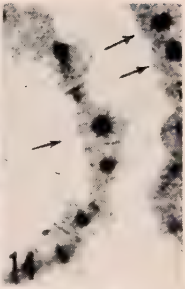
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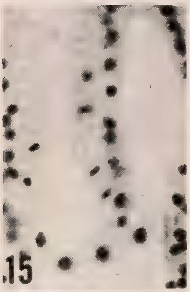
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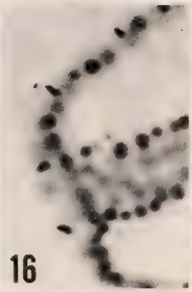
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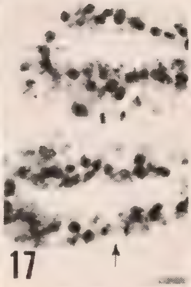
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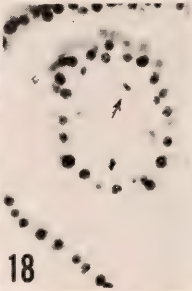
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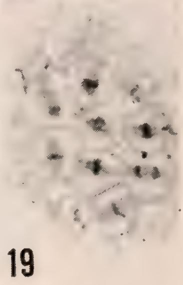
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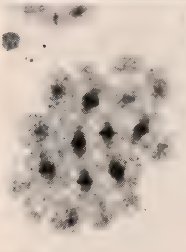
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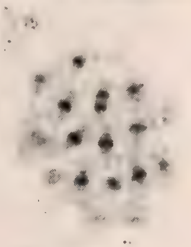
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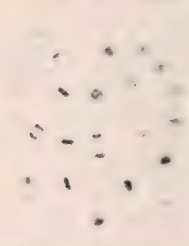
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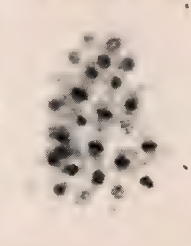
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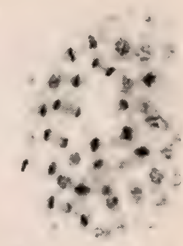
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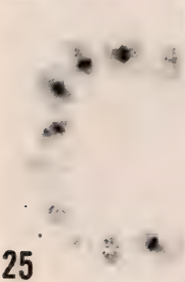
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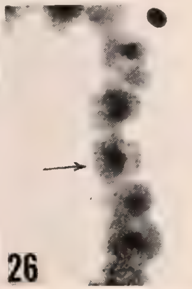
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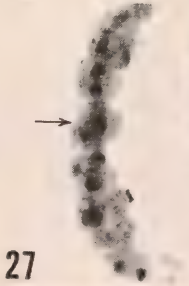
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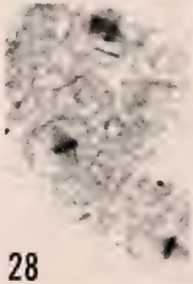
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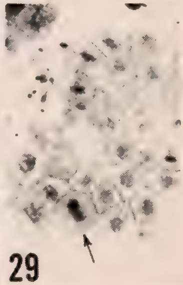
29

FIGURES 10–14. Radial sections of early, of mid-, and of late blastulae, showing spindles aligned for radial cleavage (compare Fig. 1, cells *A*, *B*, *C*). Most spindles are in equatorial view, but there are several in oblique aspect. In Figure 14, the arrows point to spindles seen in polar view.

FIGURES 15–17. Median longitudinal sections of gastrulae, showing radial cleavages. In Figures 15 and 17, anaphasic or telophasic spindles project toward the archenteron, above the level of the nuclei in other cells of the endoderm. In Figures 16 (above) and 17 (see arrow), mitotic figures of the ectoderm project out beyond the other nuclei. This phenomenon is obscured in Figure 17 due to a mild distortion, presumably resulting from pressure of another at the same level as the interphase nuclei of the adjoining cells. Metschnikoff (1885) indicated this displacement in several of his drawings, and Wolpert and Gustafson (1961) published a photomicrograph of the phenomenon occurring in a living cell (their Fig. 4).

Despite the difficulties just enumerated, the positions of most mitotic figures could be determined satisfactorily: the spindle lies parallel to the plane of the cell layer, *i.e.*, it determines a cleavage perpendicular to the cell sheet. Even cells lying in the curved surface at the blastopore cleave at right angles to the tangent at that point (Fig. 16). The same orientation prevails at the opposite end of the archenteron.

The possibility of cleavage along the long axis of the blastomere

Later blastulae occasionally include a mitotic cell in which the tangential axis is shorter than the radial. Since the spindle axis lies tangentially, as expected, the cleavage furrow in such a cell would have been radial, and therefore would have followed the longer cell diameter. If, despite fixation, dehydration, etc., this is a true picture of the cell, then the cleavage in such cases is a violation of Hertwig's rule (Wilson, 1925, p. 984).

Paraffin sections are somewhat compressed during sectioning; as a consequence, only cells in certain positions may be considered as possibly undergoing longitudinal cleavage. Figure 5 shows two spherical blastomeres before sectioning. Figure 6 shows the distortion to be expected if the blastula passes the knife in the direction indicated. All blastomeres are compressed so that their long

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embryo. In Figure 16 (below), a metaphasic cell lies in the curve of the blastopore rim and shows proper orientation for a radial cleavage.

FIGURE 18. Transverse section of a gastrula showing a metaphasic spindle lying toward the archenteron (below). The arrow points to what is really a slightly oblique polar view of a potential radial cleavage; one of the chromosome clusters (above) is somewhat out of focus. Two additional mitotic cells are out of focus but still visible, one at the left and one at the right in the endoderm.

FIGURES 19–24. Tangential sections of early and later blastulae and gastrulae, showing spindles oriented for radial cleavage (compare Fig. 2, cells *A, B, C*). An additional level of orientation is evident in Figures 20, 21, and 23, wherein most of the spindles are aligned relatively parallel to each other (see text).

FIGURE 25. Possible misinterpretation of spindle orientation. All mitotic figures, except the one directly above, are *seemingly* aligned for tangential cleavages. This interpretation, however, is incorrect, and is due to their being in a section which lay adjacent to the side of the blastula, as is indicated by the portion of a cell lying just inside the circle of cells. These cleavages are really radial (compare Figs. 3, 4, and see text).

FIGURES 26, 27. The arrows point to radial cleavages which would cut through a longer cell diameter (see Figs. 5–7). In Figure 26, the arrow also points to the bounding membrane (faint line) on the left of the blastomere. The cell above, in Figure 26, shows a pseudo-tangential division similar to those in Figure 25.

FIGURES 28, 29. Longitudinally dividing blastomeres seen in tangential sections of the blastulae. Despite its being in anaphase, the spindle of the cell in Figure 29 would lead to a cleavage along the longer diameter. The sections of Figures 26–29 were all cut as the block passed the knife from side to side (with reference to the page; see Figs. 5–7).

Staining: Figures 10, 11, 17, 23, 24, 27 were stained with gallocyanin; 12, 13, 14, 19, 20, 21, 25, 26, 28, 29 with hematoxylin; 15, 16, 18, 22 with azure A-Schiff.

Magnification: Figures 13, 14, × 480; Figure 19, × 300; Figure 27, × 550; Figure 28, × 775; all remaining, × 400.

axes are parallel to the knife edge. Cell *B* appears to be cleaving lengthwise; this, however, is obviously a pressure artifact, because the cell was spherical before sectioning (compare Figs. 5 and 6). On the other hand, cell *A* would cleave along what appears to be its shorter, *i.e.*, its radial axis. But if, despite sectioning pressure, a mitotic cell in position *A* still has a longer radial diameter, and if its spindle is tangentially oriented, then it is reasonable to assume that the cell was cleaving along a longer axis at the time of fixation (Fig. 7). Cells located between *A* and *B* could also be interpreted as dividing lengthwise as long as their spindles were oriented roughly parallel to the knife edge. Figures 26–29 are examples of such cells.

The three diameters of the blastomere, two tangential and one radial (Fig. 8), may all be unequal in length, with the radial being longest. If this condition obtains, then lengthwise cleavage may appear in either of two forms. These are shown in Figure 8 which represents such a blastomere. The division plane may follow the shortest, and one of the longer cell axes (horizontal cleavage plane, *H*, Figs. 8, 9); or the division plane may follow both longer axes (vertical cleavage plane, *V*). Since only two cell axes lie in the plane of a paraffin section, with the third projecting into the depth dimension, it is virtually impossible to determine which of these two types of planes is exemplified by a particular cleavage furrow. It is evident from Figure 9, however, that the tangential diameters may be compared on a tangential section of the blastula. Figures 28 and 29 are face views of lengthwise cleavages. Here again it is impossible to determine whether the depth diameter (radial diameter) is longer or is shorter than the two tangential diameters.

Inferences based on estimated or measured dimensions of blastomeres in paraffin sections are open to serious question. This is especially critical since cells undergo a decided change in shape during division (Wolpert and Gustafson, 1961; see their Figure 4, and their p. 381). Addition of a fixing fluid, subsequent processing, or both, might prevent a typical shape change or might produce distortions of the normally cleaving cell. Whatever the dangers of interpretation from fixed material may be, it is interesting that a suggestion of lengthwise cleavage may be observed in blastomeres as late as anaphase (Fig. 29).

General Discussion

Wolpert and Gustafson (1961) stressed the role of radial cleavage in generating and in maintaining a single layer of cells. This "first order" of patterning, however, merely restricts the number of layers to one. Other organisms, also built of single layers, may take the form of tubes or of bladders, sometimes with opposite sides appressed together (see Bonner, 1952; his Figure 79 and p. 24). It seems reasonable to infer, then, the existence of an additional level of mitotic spindle orientation which is responsible for the sub-spherical shape of the blastula.

The initial divisions of the zygote, alternating between "meridional" and "equatorial," show a pronounced regularity only to the 8-, to the 16-, or to the 32-cell stage (Seeliger, 1892; Boveri, 1901; Korschelt, 1936). Nevertheless, a single-layered sphere could be developed from this early pattern, if subsequent divisions were to "average out" in various directions around the surface.

It is not clear how this averaging might be accomplished. Completely random divisions (cleavages distributed equally among all possible radial planes cutting through the sphere) could maintain a spherical blastula. Such a pattern, however, may not actually occur. Many sections show a surprising number of spindle axes aligned in essentially the same direction (Figs. 20, 21, 23); frequently, there is a suggestion of two, mutually perpendicular systems of cleavages (Fig. 19), yet other arrangements vaguely suggest some further type or types of ordering. These may all be manifestations of another mechanism, namely, a complex, overall plan of subdivision which, while somewhat variable about the surface of the blastula, is definite and consistent at the level of the whole embryo.

Although either of the two systems of cleavage just described could generate a spherical blastula after the initial divisions, the second type is likely to prevail, at least in a general form, after invagination begins. The gastrula shows a decided elongation, which may depend on a significantly higher number of cleavages transverse to the length axis of the embryo. These would yield pairs of daughter cells aligned lengthwise, thereby increasing the length dimension. Many mitotic figures, not only in the ectoderm but in the gastrocoel wall as well, display the required orientation for transverse cleavage (Figs. 15, 16, 17).

The concept of an additional degree of mitotic spindle orientation in the blastula and in the gastrula could be confirmed by observation. If it is established, then the following questions become meaningful: what mechanism or mechanisms control spindle orientation, and how does the mechanism or the mechanisms operate? Beyond noting that the organism as a whole exerts control over its developing parts, there seems to be little that can be said in answer to such questions at the present time.

SUMMARY

Blastulae and gastrulae of the starfish, *Asterias forbesi*, were fixed, run into paraffin, sectioned, and stained to show the mitotic figures. These were found to lie in a tangential direction, thus leading to radial cleavages in the blastulae, and to their equivalent (*i.e.,* cleavage perpendicular to a tangent to the curved surface) in the gastrulae. The single layer of cells characterizing these stages is therefore the result of spindle figure and of cleavage plane orientations. Other observations suggested that the spindles may lie in definite arrangements relative to each other. Several patterns were found, and their relationship to blastula and to gastrula formation is briefly discussed. There were indications that the blastomeres may, though rarely, undergo cleavage along a longer diameter.

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